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12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

Estrogens play important roles in both the onset and malignant progression of breast cancer. The content of estrogen receptors in breast tumors is a valuable predictor of whether a patient will respond to therapy with antiestrogens, such as tamoxifen and fulvestrant (ICI 182,780). Expression and activity of ER can be lost or impaired in antiestrogen-resistant breast cancer. The proposed studies are designed to test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics. This study on mechanisms that regulate ER levels and activity are highly relevant to the development and progression breast cancer, including tumor progression to states of hormone independence and antiestrogen resistance. Thus, understanding how the estrogen receptor is regulated is an area of research critical to understanding the tissue selective pharmacology of estrogens. In addition, tamoxifen and other selective estrogen receptor modulators target the estrogen receptor, and this study is of the utmost relevance to those important therapies.

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INTRODUCTION: (Briefly, one paragraph, describe the subject, purpose and scope of the research)

Estrogen regulates diverse biological processes through estrogen receptors (ER α and ER β) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen, and receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses (2). Furthermore, several experimental results have recently demonstrated that receptor degradation is a key component of the response of cancer cells, including breast cancer cells, to antiestrogen therapy (3-5). In advanced stage breast cancers, estrogen receptor expression and activity can be lost or impaired, and the tumors are often resistant to endocrine therapies, such as the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (6, 7). Our findings during the funding period have raised the intriguing possibility for a role of ubiquitin and ubiquitin-like pathways, including the NEDD8 pathway, in ER α ubiquitination and degradation and suggest that disruptions in such pathways may contribute to the development of antiestrogen-resistance in human breast cancer. This proposal will continue to test the overall hypothesis that the ubiquitin protein modification pathways repress estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of these pathways may prove to be a valid target for novel therapeutics.

BODY (describe the research accomplishments associated with each task outlined in the approved Statement Of Work)

To address the first task of determining the effect of Uba3 on breast cancer cell proliferation, we attempted to generate a stable breast cancer cell expressing a dominant negative Uba3 (C216S), a mutant that we had used previously to block the NEDD8 pathway (8, 9). However, we were unable to generate breast cancer cells expressing this potent mutant (data not shown). Apparently, blocking this pathway is lethal and the cells die. Thus, we will use an inducible promoter as a way to control expression of C216S levels. Those experiments are underway.

The second task of the project was to determine the molecular mechanisms of ER α corepression by the NEDD8 pathway. Toward this goal, we constructed Uba3 deletion constructs lacking one or both of the presumptive nuclear receptor interacting motifs (the NR boxes). Protein-protein interaction studies were performed, using GST-pulldown assays and x-ray crystallography studies were conducted to determine which receptor domains mediate the interactions between ER α with Uba3. We were unable to detect direct interaction of the deletion mutant constructs with estrogen receptor (data not shown). However, this could be due to important changes in protein conformation due to the removal of amino acid sequences. Thus, we have taken an alternative approach and are generating point mutations within the NR boxes. The new constructs will be examined for direct interactions with ER.

As completion of the items in task 3 was reported during the last progress period, we continued to perform further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens. This was deemed a logical extension of the SOW and within the scope of the fundamental questions underlying the SOW. Thus, the role of the ubiquitin-proteasome pathway in ER α -mediated transcriptional responses in breast cancer cells

was investigated. Genetic and pharmacologic approaches were utilized to disrupt ER α ubiquitination, proteasome-mediated proteolysis and thus ER α degradation, including a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (8, 9), the 20S proteasome inhibitor MG132, a ubiquitin mutant with all of its lysines mutated to arginine (UbK0) (10, 11), and the partial agonist/antagonist tamoxifen. To determine the effect of blocking ER α degradation on estradiol-induced transcriptional responses, estrogen receptor-responsive reporter assays and expression of endogenous ER-target genes in MCF7 human breast cancer cells were utilized.

The results of this study are described in the attached manuscript (10), and some of the key findings are highlighted here. We show that proteasomal degradation is not essential for transcriptional activity of ER α and suggest that the ubiquitin-proteasome system functions to limit estradiol-induced transcriptional output. The results demonstrate that blocking polyubiquitination of ER α stabilizes the receptor, resulting in the prolonged expression of ER α -responsive genes (Figure 1B,C). Inhibiting the proteasome enhanced ER α transcriptional activity in MCF7 human breast cancer cells (Figure 5A,B), indicating that ER α degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells. The results further suggest that in cells containing low levels of ER α , proteasome-mediated receptor degradation plays a role in limiting estradiol-induced transcriptional responsiveness (Figure 1B). While blocking ER α degradation increased the magnitude of estradiol-induced gene transcription, no effect on hormone sensitivity was observed (Figure 2). However, inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ER α -target gene in breast cancer cells (Figure 5A). Overall, the data support the hypothesis that proteasome-mediated degradation of ER α serves as a means to limit the duration of estradiol signaling in receptor positive breast cancer cells. The important implication of this study is that the estradiol-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to estradiol in breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Showed that inhibiting the proteasome enhances ER α transcriptional activity in MCF7 human breast cancer cells, indicating that ER α degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells.
- Demonstrated that inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ER α -target gene in breast cancer cells.
- Demonstrated that blocking polyubiquitination of ER α stabilizes the receptor and prolongs expression of ER α -responsive genes.
- Determined that proteasomal degradation is not essential for transcriptional activity of ER α and that the ubiquitin-proteasome system appears to function to limit estradiol-induced transcriptional output.
- The data show that the estradiol-induced transcriptional response appears to be limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to estradiol in breast cancer cells.

REPORTABLE OUTCOMES (List reportable outcomes that have resulted from this research)

Manuscripts

*Fan M, Nakshatri H, **Nephew KP** Inhibiting proteasomal proteolysis sustains estrogen receptor- α activation Mol Endocrinol (in press; attached) *This award is acknowledged in this publication

Presentations

- 1) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness. Abstract 4899, 94th AACR Annual Meeting of the American Association for Cancer Research (poster/discussion)
- 2) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness. Abstract 154; Nuclear Receptors: Steroid Sisters, Keystone Symposium, Keystone, CO
- 3) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated degradation of estrogen receptor- α in estrogen-induced transcriptional response. Elwood Jensen Symposium on Nuclear Receptors and Endocrine Disorders. University of Cincinnati, Cincinnati, OH
- 4) Fan M, Nakshatri H, **Nephew KP** 2004. Uncoupling estrogen receptor- α transcriptional activity from receptor degradation 2nd Biannual Midwest Regional Molecular Endocrinology Conference, Indianapolis, IN (platform talk)

CONCLUSIONS

In target tissues where ER α levels are limiting, the magnitude of the response to estradiol is correlated with cellular ER α concentrations (2, 12). The ubiquitin-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating estradiol levels. Our results indicate that the magnitude and duration of estradiol-induced gene transcription are limited by proteasome-mediated degradation of ER α ; therefore, it seems reasonable to speculate that defects in ER α -degradation could lead to enhanced cellular responsiveness to estrogens. In support, aberrant ER α expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (13-15), and during the previous project period we reported that blocking ER α degradation rendered breast cancer cells insensitive to the growth inhibitory effects of ICI 182,780, a potent ER α downregulator (9). We will attempt to elucidate whether defects in the ER α degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance.

For the "so what section" (evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report), the loss of ER α degradation pathway(s) may provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining

expression of ER α . Pathways that utilize the ubiquitin-proteasome system could serve as a therapeutic targets for breast cancer.

In summary, Tasks 1 and 2 are in progress. Task 3 has been completed but extended to include further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens.

List of personnel receiving pay from the research effort: Kenneth P. Nephew, Ph.D., Principal Investigator; Meiyun Fan, Ph.D., Postdoctoral Fellow; Teresa Craft, M.S., Research Associate

REFERENCES CITED

1. McKenna NJ, O'Malley BW 2001 Nuclear receptors, coregulators, ligands, and selective receptor modulators: making sense of the patchwork quilt. *Ann N Y Acad Sci* 949:3-5
2. Webb P, Lopez GN, Greene GL, Baxter JD, Kushner PJ 1992 The limits of the cellular capacity to mediate an estrogen response. *Mol Endocrinol* 6:157-67
3. Wijayaratne AL, McDonnell DP 2001 The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 276:35684-92
4. Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS 2004 Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res* 64:1522-33
5. Laios I, Journe F, Laurent G, Nonclercq D, Toillon RA, Seo HS, Leclercq G 2003 Mechanisms governing the accumulation of estrogen receptor alpha in MCF-7 breast cancer cells treated with hydroxytamoxifen and related antiestrogens. *J Steroid Biochem Mol Biol* 87:207-21
6. Klinge CM 2000 Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 65:227-51.
7. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Mol Endocrinol* 2:1157-62
8. Wada H, Yeh ET, Kamitani T 2000 A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. *J Biol Chem* 275:17008-15
9. Fan M, Bigsby RM, Nephew KP 2003 The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the antiproliferative activity of ICI 182,780 in ERalpha-positive breast cancer cells. *Mol Endocrinol* 17:356-65
10. Fan M, Nakshatri H, Nephew KP. Inhibiting proteasomal proteolysis sustains estrogen receptor- α activation *Mol Endocrinol* (in press)
11. Bloom J, Amador V, Bartolini F, DeMartino G, Pagano M 2003 Proteasome-mediated degradation of p21 via N-terminal ubiquitylation. *Cell* 115:71-82
12. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y 1987 Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 47:4355-60
13. Clarke RB, Howell A, Potten CS, Anderson E 1997 Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 57:4987-91

14. Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, Sibson DR, Sloane JP 1999 Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *Am J Pathol* 155:1811-5
15. Shoker BS, Jarvis C, Clarke RB, Anderson E, Munro C, Davies MP, Sibson DR, Sloane JP 2000 Abnormal regulation of the oestrogen receptor in benign breast lesions. *J Clin Pathol* 53:778-83

APPENDICES

Copy of Manuscript:

Fan M, Nakshatri H, **Nephew KP** Inhibiting proteasomal proteolysis sustains estrogen receptor- α activation *Mol Endocrinol* (In Press; see attached)

Copies of Abstracts

- 1) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness. Abstract 4899, 94th AACR Annual Meeting of the American Association for Cancer Research (poster/discussion)
- 2) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness. Abstract 154; Nuclear Receptors: Steroid Sisters, Keystone Symposium, Keystone, CO
- 3) Fan M, Nakshatri H, **Nephew KP** 2004. Uncoupling estrogen receptor- α transcriptional activity from receptor degradation 2nd Biannual Midwest Regional Molecular Endocrinology Conference, Indianapolis, IN (platform talk)

Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor- α Activation

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The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; csFBS, dextran-coated charcoal-stripped fetal bovine serum; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; hnRNA, heterogeneous nuclear RNA; luciferase, firefly luciferase; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; SRC, steroid receptor coactivator; Ub, Ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin

Key Words: estrogen receptor, proteasome, transactivation, degradation

20 Abstract

Estrogen receptor- α (ER α) is a ligand-dependent transcription factor that mediates physiological responses to 17 β -estradiol (E2). Ligand binding rapidly down-regulates ER α levels through proteasomal proteolysis, but the functional impact of receptor degradation on cellular responses to E2 has not been fully established. In this study, we investigated the effect of blocking the ubiquitin-proteasome pathway on ER α -mediated transcriptional responses. In HeLa cells transfected with ER α , blocking either ubiquitination or proteasomal degradation markedly increased E2-induced expression of an ER-responsive reporter. Time course studies further demonstrated that blocking ligand-induced degradation of ER α resulted in prolonged stimulation of ER-responsive gene transcription. In breast cancer MCF7 cells containing endogenous ER α , proteasome inhibition enhanced E2-induced expression of endogenous pS2 and cathepsin D. However, inhibiting the proteasome decreased expression of progesterone receptor (PR), presumably due to the heterogeneity of the PR promoter, which contains multiple regulatory elements. In addition, in endometrial cancer Ishikawa cells overexpressing coactivator SRC-1, 4-hydroxytamoxifen displayed full agonist activity and stimulated ER α -mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ER α transcriptional activity and functions to limit E2-induced transcriptional output. The results further indicate that promoter context must be considered when evaluating the relationship between ER α transcription and proteasome inhibition. We suggest that the transcription of a gene driven predominantly by an estrogen responsive element, such as pS2, is a more reliable indicator of ER α transcription activity than a gene like PR, which contains a complex promoter requiring cooperation between ER α and other transcription factors.

INTRODUCTION

The actions of estrogens are mediated primarily through estrogen receptors (ER α and ER β) (1), ligand-dependent transcription factors that interact directly with estrogen response elements (EREs) in the promoters of target genes (1). Cellular levels of ER α (2), along with a large number of receptor coregulator complexes (3), play key roles in controlling appropriate physiological responses in estrogen target tissues, such as breast and uterus. Levels of ER α mRNA and protein are regulated primarily by its cognate ligand, 17 β -estradiol (E2) (4-6). E2 binding results in rapid turnover of ER α protein through the ubiquitin-proteasome pathway (7-11), which has been implicated in both the overall control of gene transcription (12-16) and transactivation function of ER α and other nuclear receptors (7, 17-24).

The ubiquitin-proteasome system consists of the 26S proteasome, a complex composed of a 20S catalytic core for protein proteolysis and two ATPase-containing 19S regulatory particles that recognize polyubiquitin-tagged substrates (25). Like many other transcription factors, stimulation of ER α transcriptional activation appears to be associated with receptor ubiquitination and proteasomal degradation (11, 26). Several proteins possessing ubiquitin ligase activity (e.g., E6AP, p300, BRCA1, and MDM2), as well as SUG1, a component of the 19S proteasome, have been shown to associate with ER α and modulate receptor signaling (27-34). These observations suggest that proteasome-mediated receptor degradation is important for ER function.

Recent studies have demonstrated that inhibiting proteasomal degradation increases transcriptional activity of many, but not all, nuclear receptors, indicating a receptor-specific effect of proteasome inhibition (17-24). Blocking ER α turnover by a proteasome-specific inhibitor, MG132, results in decreased expression of an ER α -responsive luciferase reporter,

implicating that proteasomal degradation of ER α is required for its transactivation function (7, 35). However, MG132, and other proteasome inhibitors, have recently been shown to deleteriously affect on production of a functional firefly luciferase enzyme (36), complicating the assessment of studies utilizing only ER α -responsive reporters expressing luciferase, in combination with 20S proteasome inhibitors. In addition, several studies have recently suggested that receptor degradation may not be required for ER α -mediated transcription. Frasor *et al.* reported that the partial agonist/antagonist 4-hydroxytamoxifen (4-OHT), which protects ER α from proteasomal degradation (11, 37), stimulates ER-mediated transcription of a group of genes in MCF7 cells (38). Dissociation of ER α activation from degradation has also been reported in pituitary tumor cells (39, 40).

In the present study, we investigated the role of the ubiquitin-proteasome pathway in ER α -mediated transcriptional responses. Genetic and pharmacologic approaches were utilized to disrupt ER α ubiquitination, proteasome-mediated proteolysis and thus ER α degradation, including the 20S proteasome inhibitor MG132, a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (41, 42), a ubiquitin mutant with all of its lysines mutated to arginine (UbK0) (43), and the partial agonist/antagonist 4-OHT. To determine the effect of blocking ER α degradation on E2-induced transcriptional responses, ER-responsive reporter assays and expression of endogenous ER-target genes were utilized. The results of this study demonstrate that proteasomal degradation is not essential for transcriptional activity of ER α and indicate that the ubiquitin-proteasome system functions to limit E2-induced transcriptional output.

RESULTS

Inhibiting the proteasome increases ER α transcriptional output

90 The enzymatic activity of chloramphenicol acetyltransferase (CAT), luciferase (Luc) or
 β -galactosidase (Gal) reporter proteins is commonly used for assessing transcriptional activity of
 nuclear receptors in the presence of proteasome inhibitors. Recent studies with breast cancer
 T47D cells revealed that proteasome inhibitors (MG132, lactacystin and proteasome inhibitor I)
 interfere with the production of luciferase and galactosidase proteins by a post-transcriptional
95 mechanism, while the enzymatic activity of CAT remains unaffected (36). To verify these
 observations in our experimental systems, we examined the effect of MG132 on expression of
 these reporter enzymes from constitutively active constructs in cervical carcinoma HeLa and
 breast cancer MCF-7 cells. Cells were transfected with RSV-CAT, SV40-Luc or pCMV- β -gal
 and then treated with vehicle (DMSO) or MG132 (1 μ M) for 24 h. Reporter enzyme activity
100 was determined using standard assays for luciferase, CAT and galactosidase. Treatment of HeLa
 cells with MG132 had no effect on CAT activity but decreased luciferase and galactosidase
 activity by 80% and 30%, respectively (Fig 1A, left panel). Essentially similar results were
 obtained using MCF7 cells (Fig 1A, right panel). These results agree with a previous report
 demonstrating that proteasome inhibitors have deleterious effects on the enzymatic activities of
105 luciferase and galactosidase reporter proteins (36).

 Previously, we and others showed that E2 induces ER α degradation in transiently
 transfected HeLa cells and MG132 abolishes such degradation (8, 9, 42). Based on the above
 results, we further investigated the relationship between ER α turnover and E2-induced
 transcriptional response using an E2-responsive CAT reporter. HeLa cells were transiently
110 transfected with ERE-Vit-CAT and different doses of ER α -expressing construct (0.1 – 5 ng

pSG5-ER α /10⁵ cells). Cells were treated with vehicle (DMSO) or MG132 (1 μ M) for 1 h followed by E2 (10 nM). CAT activity was measured 24 h after E2 treatment. Basal CAT activity increased, proportional to the amount of pSG5-ER α (Fig. 1B; open bars). As expected, E2 markedly induced CAT activity (Fig. 1B; gray bars); however, treatment with MG132 plus E2 resulted in greater CAT activity, compared to E2 alone (Fig. 1B; black vs. gray bars). Cells treated with MG132 alone exhibited slightly higher CAT activity than the DMSO control (Fig. 1B, hatched bars). A synergistic effect of MG132 plus E2 was observed in cells transfected with lower levels of ER α (0.1 – 0.3 ng pSG5-ER α /10⁵ cells). For example, the combined treatment of MG132 and E2 increased ERE-CAT activity by about 7.4-fold in cells transfected with 0.1 ng pSG5-ER α /10⁵ cells, whereas MG132 or E2 alone increased ERE-CAT activity by 1.82- or 3.10-fold, respectively (Table in Fig. 2B). Immunoblot analysis showed that pretreatment with MG132 effectively blocked E2-induced ER α down-regulation in HeLa cells (Fig. 1C). Taken together, these observations demonstrate that ER α retains the capacity to activate transcription in the absence of proteasomal degradation, and blocking ER α turnover increases E2-induced transcriptional output. The results further suggest that, in cells containing low levels of ER α , proteasome-mediated receptor degradation plays a role in limiting E2-induced transcriptional responsiveness.

Effect of inhibiting the proteasome on E2 sensitivity

Based on the observation that preventing receptor protein turnover increases ER α -mediated transcription, we examined the effect of inhibiting the proteasome on hormone sensitivity. HeLa cells were transfected with ERE-Vit-CAT and pSG5-ER α , treated with DMSO or MG132 for 1 h, and then treated with various doses of E2 (1×10^{-15} - 1×10^{-8} M). CAT activity

was determined 24 h after the addition of ligand. In cells transfected with 0.3 ng (Fig. 2A) or 1
135 ng pSG5-ER α (Fig. 2B), a hyperbolic dose response to E2 was observed; the lowest dose of
hormone that induced CAT activity was 1×10^{-11} M E2. Increasing ER α expression (0.3 ng vs. 1
ng pSG5-ER α) and pretreatment with MG132 augmented maximal CAT induction by E2, but no
effect on E2 sensitivity was observed. The minimal dose of E2 required to induce CAT was
 1×10^{-11} M under all experiment conditions, and the EC50 was not different (Fig. 2). These
140 results demonstrate that blocking ER α degradation increases the magnitude of E2-induced gene
transcription but has no effect on hormone sensitivity.

Inhibiting the proteasome extends the duration of E2-induced gene transcription

The results of the above experiments suggest that inhibiting the proteasome may extend
145 the half-life of ligand-activated ER α and thus increase receptor transcriptional output. To test the
possibility that MG132 treatment would subsequently extend the duration of an E2-induced
transcriptional response, we performed a time course analysis using luciferase as a reporter
protein. The half-life of CAT in mammalian cells is about 50 h (44); in contrast, luciferase has
an intracellular half-life of about 3 h (44), making it well suited for performing a dynamic
150 analysis of promoter activation. Thus, we used HeLa cells transfected with ER α and ERE-pS2-
Luc to study the effect of proteasome inhibition on E2-induced transcription in a time-dependent
manner. In transfected HeLa cells, E2 induced a transient induction of luciferase activity,
maximal at 6 h (Fig. 3A, closed circles). Pretreatment with MG132 decreased E2-induced
luciferase expression at the early time points (1.5 h to 6 h), but markedly increased E2-induced
155 luciferase expression from 9 to 20 h (Fig. 3A, closed triangles).

As mentioned above, MG132 can inhibit luciferase production. To determine the effect of MG132 on luciferase synthesis in general, we transfected HeLa cells with a constitutively active luciferase construct (SV40-Luc). In contrast to what we observed using ERE-pS2-Luc, MG132 consistently decreased the expression of SV40-Luc during the 20 h period (Fig 4B), excluding the possibility that MG132 enhances ERE-luc activity by stabilizing luciferase protein. To subtract the general inhibitory effect of MG132 on luciferase synthesis, at each time point shown in Fig. 3C, ER α -mediated luciferase expression in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct (Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 x [SV40-Luc activity/SV40-Luc activity in the presence of MG132]). The adjusted results clearly demonstrate that blocking receptor degradation with MG132 increases both the magnitude and duration of E2-induced gene transcription, suggesting that the duration of gene transcription induced by E2 is limited by ER α degradation through the 26S proteasome.

Inhibiting ER α ubiquitination prolongs E2-induced gene transcription

In a previous study, we used a dominant negative mutant of the NEDD8 conjugation enzyme, Ubc12C111S, to inhibit ER α ubiquitination and degradation (42). Here we used Ubc12C111S as a means to investigate the role of ER α turnover in ER α transactivation function and to corroborate our observations using MG132. The impact of Ubc12C111S on the time-dependent induction of a reporter gene by ER α was investigated. HeLa cells were transfected with pSG5-ER α , ERE-pS2-Luc, along with a control vector (pcDNA) or a construct expressing the mutant Ubc12 (pcDNA-Ubc12C111S). In cells transfected with pcDNA, E2 transiently induced luciferase expression, and maximal induction was observed at 5 h (Fig. 3D, closed

circles). However, in cells transfected with pcDNA-Ubc12C111S, a delay in peak expression of
180 E2-induced luciferase activity was observed (9 h; Fig. 3D, closed triangles), and luciferase
expression remained elevated, even 20 h after E2 treatment. No effect of Ubc12C111S on
maximal E2-induced luciferase activity was observed (Fig. 3D, closed circles vs. closed
triangles). To confirm that the observed effect of Ubc12C111S on ER α -mediated luciferase
expression was specific, luciferase activity in cells cotransfected with SV40-Luc and
185 Ubc12C111S was assessed over time. No effect of Ubc12C111S on SV40-Luc expression was
seen at 6 and 12 h post-transfection; a slight increase in luciferase expression was observed at 20
h (1.3-fold; Fig. 3E). Overall, these results demonstrate that inhibiting ER α ubiquitination
prolongs ER α -mediated transcription, supporting the hypothesis that proteasome-mediated
degradation of ER α serves as a means to limit the duration of E2 signaling.

190 **Blocking polyubiquitination sustains E2-induced gene expression**

To determine the effect of blocking polyubiquitination on ER α -mediated transcription, we
utilized a ubiquitin mutant, UbK0, which has all of its lysines replaced by arginine. This mutant
competes with endogenous ubiquitin and terminates ubiquitin chains, resulting in the
195 accumulation of short ubiquitin conjugates that cannot be degraded efficiently by the proteasome
(43). First, we examined the effect of overexpressing UbK0 on E2-induced ER α degradation. In
HeLa cells cotransfected with wild-type ubiquitin (Ub) and ER α , the level of receptor protein
decreased markedly after E2 treatment (Fig. 4A), accompanied by transient E2-induced
expression of an ER-responsive luciferase reporter gene (Fig. 4B, 8 h vs. 24 h). In contrast, cells
200 transfected with UbK0 showed sustained E2-induced luciferase expression (Fig. 4B), and no
decrease in ER α protein levels was observed (Fig. 4A). Furthermore, the effect of UbK0 on

ER α -induced luciferase was specific, as UbK0 showed no effect on expression of the SV40-Luc construct (Fig. 4C). These results demonstrate that blocking polyubiquitination of ER α stabilizes the receptor, resulting in the prolonged expression of an ER α -responsive gene.

205

Proteasome inhibition enhances ER α -mediated transcription in MCF7 breast cancer cells

To further investigate the role of ER α degradation in receptor transactivation ability under physiologically relevant conditions, we examined the effect of inhibiting the proteasome in MCF7 breast cancer cells, which endogenously express ER α . First, we examined the effect of
210 MG132 on ERE-Vit-CAT expression in MCF7 cells. MCF7 cells were transiently transfected with ERE-vit-CAT and then treated with DMSO or MG132 (1 μ M) for 1 h prior to E2 (10 nM) treatment. CAT activity was determined 24 h after E2 treatment. A 17.8 ± 1.7 fold increase in CAT expression was seen in MCF7 cells treated with E2, compared to the control; treatment with MG132 further increased E2-induced CAT activity to 25.6 ± 2.5 fold. Therefore, inhibiting
215 the proteasome enhanced ER α transcriptional activity in MCF7 cells, indicating that ER α degradation plays a key role in limiting E2-induced transcriptional responses in breast cancer cells.

To determine the effect of proteasome inhibition on transcription of ER α -target genes in breast cancer cells, we pretreated MCF7 cells with MG132 and examined E2-induced pS2 gene
220 expression. ER α regulates pS2 transcription through an imperfect palindromic ERE at position -405 to -393 of its promoter region (45); pS2 expression is considered a reliable indicator of ER α transcriptional activity (46). Time-dependent effects of MG132 on heterogeneous nuclear pS2 RNA (pS2 hnRNA) levels, which reflect the rates of pS2 gene transcription (47-50), were examined. Primers amplifying the conjoining sequence between the first intron and second exon

225 of the pS2 gene were used, and expression of pS2 hnRNA was assessed by real-time quantitative reverse transcription-PCR (Q-PCR). After administration of E2, levels of pS2 hnRNA increased by 3 h, peaked at 12 h, and then declined by 70% during the next 8 h (Fig. 5A, gray bars). However, at all time points examined, E2-induced expression of pS2 hnRNA was markedly enhanced by pretreatment with MG132 (Fig. 5A, black vs. gray bars), and pS2 hnRNA levels 230 declined only by 15% from 12 h to 20 h after the combined treatment (Fig. 5A, black bars). MG132 alone showed no effect on basal pS2 hnRNA expression (Fig. 5A, hatched bars). In agreement with what we observed with pS2 hnRNA, the combined treatment of MG132 plus E2 resulted in greater expression of pS2 mRNA after 6 h, compared to E2 treatment alone (Fig. 5B, black vs. gray bars); pS2 mRNA levels remained markedly elevated up to 20 h, the last time 235 point examined (Fig. 5B, black bars). The coordinate increase in E2-induced expression of both pS2 hnRNA and pS2 mRNA by MG132 excludes the possibility that MG132 inhibits the hnRNA splicing process or stabilizes pS2 mRNA. Therefore, it seems reasonable to conclude that blocking the proteasome with MG132 enhances E2-induced pS2 transcription initiation. Together, these results demonstrate that inhibiting the proteasome increases both the magnitude 240 and duration of E2-induced expression of the endogenous pS2 gene in breast cancer cells.

We also examined the effect of MG132 on mRNA expression of cathepsin D and progesterone receptor (PR), two well-known E2-regulated genes, in MCF7 cells. As shown in Fig. 5C, a transient increase in cathepsin D mRNA expression was observed after treatment with E2. Pretreatment with MG132 enhanced both basal and E2-induced cathepsin D expression at 3 245 and 6 h (Fig. 5C, black vs. gray bars); however, at 12 and 24 h, the effect of MG132 was no longer apparent. Treatment of MCF7 cells with E2 increased PR mRNA levels 7-fold by 3 h, and PR mRNA levels remained elevated throughout the experiment period (Fig. 5D, gray bars).

MG132 pretreatment decreased E2-induced expression of PR mRNA by over 50% at all time points examined (Fig. 5D, black vs. gray bars), which agrees with a recent report that MG132 inhibits ER α -induced increase in PR protein levels (7). The differential effects of MG132 on these ER α -target genes demonstrate that promoter context must be considered when evaluating MG132 regulation of ER α -mediated transcription. Immunoblotting analysis showed that pretreatment with MG132 efficiently blocked E2-induced ER α down-regulation in MCF7 cells (Fig. 5E).

4-Hydroxytamoxifen stimulates ER α -mediated transcription without inducing ER α degradation

The antiestrogen 4-OHT has been shown to up-regulate ER α levels by blocking ER α degradation (37), and previous studies have shown that 4-OHT functions as an ER α agonist in Ishikawa endometrial cancer cells (51, 52). To further examine the relationship between receptor stability and ER α -mediated transcription, we stably transfected ER α -negative Ishikawa cells with ER α . The ER α (+) Ishikawa cells were then transfected with a luciferase reporter construct containing the human C3 promoter (C3T1-Luc) and then treated with either E2 (10 nM) or 4-OHT (1 μ M) for 16 h. After E2 administration, a 2-fold increase in luciferase activity was observed (Fig. 6A), accompanied by a marked decrease in ER α protein level (Fig. 6B). Treatment with 4-OHT also stimulated expression of luciferase (80% of E2-stimulated luciferase expression) (Fig. 6A), but the antiestrogen did not down-regulate ER α (Fig. 6B). Thus, these results demonstrate that the partial agonist activity of 4-OHT and ER α degradation are not coupled in endometrial cancer cells. It has been reported that the steroid receptor coactivator SRC1, by stimulating transcription activity of 4-OHT liganded ER α (53), can convert 4-OHT to

a full agonist. We reasoned that if receptor degradation is essential for ER α to initiate transcription, SRC1 should enhance 4-OHT-stimulated ER α transactivation activity and, in parallel, induce proteasomal degradation of 4-OHT liganded ER α . To test this reasoning, the ER α (+)Ishikawa cells were cotransfected with a construct expressing SRC1 and C3T1-Luc, and
275 then treated with either E2 (10 nM) or 4-OHT (1 μ M) for 16 h. As expected, over-expressing SRC1 resulted in similar 4-OHT- and E2-stimulated ER α activity (Fig. 6A); however, 4-OHT did not induce receptor down-regulation (Fig. 6B). Thus, under these experimental conditions, 4-OHT, even when behaving as a full agonist in the presence of an increased level of SRC-1, did not induce ER α degradation. Taken together, these results demonstrate that ER α -mediated gene
280 transactivation can be uncoupled from receptor degradation.

DISCUSSION

Like other rapidly turned-over transcription factors, engagement of ER α in transactivation is coupled to ER α degradation by the ubiquitin-proteasome pathway (7-11, 35). However, the functional impact of ER α degradation on cellular responses to E2 has not been well established. In this study, we analyzed the effect of blocking ER α degradation on E2-induced transcriptional output. We demonstrate that blocking ER α turnover prolongs the ability of ER α to transactivate target genes and increases the output of E2-induced gene transcription. We also show that 4-OHT can act as a full agonist in Ishikawa cells overexpressing SRC-1 to stimulate ER α transcriptional activity, without inducing receptor degradation. Furthermore, proteasome inhibition by MG132 increases ER α -mediated reporter gene expression, as well as expression of endogenous ER α -target genes (pS2 and cathepsin D), in MCF7 breast cancer cells. These data demonstrate that proteasomal degradation is not essential for ER α transcriptional activity; ER α remains functional after escaping ubiquitination and proteasomal proteolysis. An important implication of this study is that the E2-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ER α could lead to an enhanced cellular response to E2.

In this study, several approaches targeting different steps in ubiquitination/proteasome proteolysis were utilized to block ER α degradation. MG132 was used to inhibit ER α proteolysis by specifically blocking activity of the 20S proteasome. A dominant negative mutant (Ubc12C111S) of the NEDD8 conjugation enzyme was used to block ER α ubiquitination by inhibiting ubiquitin ligase activity (41, 42). A ubiquitin mutant with all of its lysines mutated to arginine (UbK0) was used to block ER α polyubiquitination by terminating polyubiquitin chains (43). One concern regarding the use of these approaches is a lack of specificity, such that the

observed effect on enhanced E2-induced transcriptional output could be due to stabilization of multiple regulatory proteins, in addition to ER α . However, several observations suggest that this is not the case. MG132, Ubc12C111S and UbK0 substantially enhance E2-induced, but not basal, expression of ERE-reporter genes or the endogenous pS2 gene, suggesting that the effect of these inhibitors on ER α target gene expression is hormone-dependent and thus receptor-dependent. Furthermore, a time-dependent effect on E2-induced gene transcription was observed, which agrees with the ability of these inhibitors to block ligand-induced ER α degradation. Finally, no time-dependent effect on SV40-Luc expression was observed, in contrast to ERE-Luc, suggesting that these inhibitors do not broadly affect gene transcription in a time-dependent manner. Therefore, we conclude that MG132, Ubc12C111S and UbK0 enhance E2-induced gene transcription primarily by extending the lifetime of functional ER α .

Consistent with our ER α findings, proteasome inhibition has been shown to enhance the transcriptional response mediated by other nuclear receptors, including the glucocorticoid receptor (GR) (17, 24), aryl hydrocarbon receptor (18), peroxisome proliferator-activated receptor α (19), retinoid receptors (20) and the vitamin D3 receptor (21). However, it has also been reported that MG132 decreases transcriptional activity of PR and androgen receptor (AR) (22, 23), indicating that the effect of proteasome inhibition on transcriptional activity could be receptor-specific. This is presumably due to the involvement of mechanisms other than modulation of receptor levels; for example, MG132 inhibited AR activity by eliminating androgen-induced nuclear translocation and coactivator recruitment (22, 23).

In MCF7 cells, we observed differential effects of MG132 on E2-induced transcription of endogenous pS2, cathepsin D and PR gene, suggesting that proteasome inhibition can have promoter-specific effects on gene transcription. While the reason for this is not clear, these

observations raise the intriguing possibility of a differential requirement of ER α turnover in gene transcription, such that ER α degradation is required for PR transcription, but not for pS2 and cathepsin D. However, another attractive possibility is that multiple regulatory elements, other than an ERE, could be differentially regulated by proteasome inhibition; the different structures of the PR, pS2 and cathepsin D promoters may favor this possibility. For endogenous genes, the effect of estrogen is usually mediated through crosstalk between the ERE and nearby regulatory elements, and there appears to be an inverse correlation between the influence of nearby elements and the "strength" of the ERE (54). The ERE sequence in pS2 promoter deviates from the consensus palindromic ERE by 1 base pair (bp) and, when isolated from surrounding sequences, is able to mediate estrogen responsiveness (45); however, for the cathepsin D promoter, although the ERE-like sequence deviates from the consensus ERE by only 2 bp, it is unable to confer estrogen regulation alone and must cooperate with other regulatory elements (54). In the case of the PR promoter, only a half-site ERE is found, and estrogen induction of PR appears to require cooperation with nearby Sp1 and AP-1 sites (55). Based on the observation that ERE-vit-CAT (Fig. 1 B) and ERE-pS2-Luc (Fig. 2) activities correlate with cellular concentrations of ER α , we suggest that ER α levels are the determining factor for the transcription activity of genes controlled exclusively by ERE. We further suggest that transcriptional activity of endogenous genes driven predominantly by an ERE (e.g., pS2) may depend upon the availability of ER α . In contrast, the level of ER α is unlikely to be the sole determining factor for the transcription of genes without a consensus ERE in their complex promoters (e.g., PR). In support of this notion, it has been reported that E2-induced transcription of the PR gene does not parallel ER α occupancy (55). Therefore, it is possible that MG132 inhibits PR expression through other protein factors, either directly or indirectly. In this respect,

350 when evaluating the transcriptional activity of ER α , after escaping proteasome degradation,
promoter context must be considered. Based on our and others' results (50), it is plausible that
the transcription rate of a gene driven predominantly by an ERE is a more reliable "readout" of
ER α transcription activity than a gene containing a complex promoter requiring ER α plus other
transcription factors.

355 Our results differ from a previous study by Reid *et al.* (35), showing that MG132
prevented recruitment of phosphorylated RNA pol II (p-Pol II) to the pS2 promoter. This is most
likely due to different experimental conditions and endpoints used in the two studies. For
example, Reid *et al.* used a higher dose (10 μ M) and longer pretreatment (7 h) with MG132 in
their study. However, under that condition, it is not clear whether the drug had any effect on p-
360 Pol II recruitment to non-estrogen responsive promoters. In addition, although α -amanitin was
used to "clean" the pS2 promoter before p-Pol II recruitment analysis, it is not clear that gene
transcription resumed immediately (within a 2 h period) after α -amanitin treatment. Thus,
whether the differential recruitment of p-Pol II, in the absence or presence of MG132 following
 α -amanitin pretreatment, is correlated with pS2 gene transcription remains an open question.

365 However, the observation by Reid *et al.* (35) that the 20S proteolytic subunit does not associate
with the pS2 promoter in response to E2 stimulation, agrees with numerous studies showing that
the 20S proteasome subunit is not required for transcription initiation and elongation (56-60).
Our observation further shows that 20S proteasome activity is not essential for ER α -mediated
gene transcription.

370 Although the mechanism(s) by which the proteasome modulates ER α -mediated
transactivation remains to be fully elucidated, chromatin immunoprecipitation assays have
demonstrated that both unliganded and liganded receptors constantly cycle on and off estrogen-

responsive promoters (35). MG132 appears to halt this cyclic interaction, leading to prolonged occupancy of ER α on EREs (35). The cyclic turnover of ER α could be a mechanism used by cells to prevent multiple rounds of transcription initiation from a single promoter, thus ensuring an appropriate cellular response to changes in circulating concentrations of hormone. To support this explanation, recent studies of GR show that proteasome inhibition dramatically increases both the residence time of GR on its target promoter and transcriptional output (24). In addition to extending the half-life of ligand-activated ER α , other factors, such as increased cellular concentration of receptor coactivators, could contribute to the enhancement of transcription by proteasome inhibition. Several ER α coactivators, including the steroid receptor coactivator family members (SRC1, SRC2 and SRC3) and CREB-binding protein (CBP/p300), are substrates of proteasomal degradation; proteasome inhibition appears to increase cellular concentrations of these coactivators (61).

We found that blocking ER α degradation (using MG132, Ubc12C111S or UbK0) decreases E2-induced ERE-pS2-Luc expression at earlier time points (1.5 - 6 h) following E2 treatment (Fig. 3 and 4). While the reason for this is unknown, one possibility is that ubiquitination and 20S proteasome activity are required for optimal ER α activation, perhaps by facilitating the release of ER α from pre-existing corepressor complexes. In order to fully elucidate the physiological role(s) of ubiquitination, identification of the primary ubiquitin ligase(s) for ER α , as well as the ubiquitination site(s) in this receptor, will be necessary.

In target tissues where ER α levels are limiting, the magnitude of the response to E2 is correlated with cellular ER α concentrations (2, 62). The ubiquitin-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating E2 levels. Our results indicate that both the magnitude and duration of

E2-induced gene transcription are limited by proteasome-mediated degradation of ER α ; therefore, it seems reasonable to speculate that defects in ER α -degradation could lead to enhanced cellular responsiveness to estrogens. In support of this possibility, it has been demonstrated that thyroid hormone and insulin, by blocking ligand-induced ER α degradation,

400 can augment E2-stimulated cell proliferation (39, 63). Therefore, our future studies will examine the functional impact of proteasome-mediated ER α degradation on complex biological responses to estrogens, such as mammary gland development. In addition, aberrant ER α expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (64-66).

Our previous studies demonstrate that blocking ER α degradation render breast cancer cells
405 insensitive to the growth inhibitory effects of ICI 182,780, a potent ER α downregulator (42).

Whether defects in the ER α degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance remains to be elucidated.

MATERIALS AND METHODS

410 Plasmid Construction

The construction of pSG5-ER α (HEGO), ERE2-pS2-Luc, pcDNA-HA-Ubc12C111S, C3T1-Luc, pcDNA-SRC1, pCS2-UbK0 and ERE-vit-CAT has been described previously (43, 67, 68).

Cell Lines

415 The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). The ER α -negative endometrial Ishikawa cell line was kindly provided by Dr. S. Hyder (University of Missouri, Columbia). HeLa and Ishikawa cells were maintained in minimum essential medium (MEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 units/ml penicillin, 420 50 μ g/ml streptomycin, and 10% FBS. MCF7 cells were maintained in the same medium with the addition of 6 ng/ml insulin. Prior to experiments involving hormone treatment, cells were cultured in hormone-free medium (phenol red free MEM with 3% dextran-coated charcoal-stripped FBS (csFBS)) for 3 days.

425 Transient Transfection and Reporter Enzyme Assays

Cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium. Unless stated otherwise, 430 24 h after transfection, cells were treated with vehicle (DMSO) or MG132 (Sigma Chemical Co., St. Louis, MO) for 1 h prior to E2 (Sigma) treatment. At the end of the experiment, cell lysates

were prepared for reporter enzyme assays. Luciferase activity was determined using the Luciferase Assay System (Promega Corp., Madison, WI), Gal activity was determined using a chemiluminescent reporter assay (PE Applied Biosystems, Foster City, CA) and CAT activity
435 was determined using the colorimetric CAT ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Total cellular protein was determined by using Protein Assay Kit (Bio-Rad laboratories Inc., Hercules, CA). Reporter activities were expressed as relative light units normalized to total cellular protein.

440 **Quantitative Real Time PCR (Q-PCR)**

MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 days. The cells were pretreated with MG132 (5 μ M) for 1 h prior to E2 (10 nM) treatment. Total RNA was prepared by a RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA (2 μ g) was reverse-transcribed in a total volume
445 of 40 μ l containing 400 units M-MLV (New England Biolabs, Beverly, MA), 400 ng random hexamers (Promega), 80 units RNase Inhibitor and 1 mM dNTPs. The resulting cDNA was used in subsequent Q-PCR reactions, performed in 1x iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers. The primers used in the Q-PCR were, for pS2 mRNA: forward primer, 5'-ATACCATCGACGTCCCTCCA-3' and reverse primer, 5'-

450 AAGCGTGTCTGAGGTGTCCG-3' (69); for pS2 hnRNA: forward primer, 5'-TTGGAGAAGGAAGCTGGATGG -3' (start position 3997, within the intron); reverse primer, 5'- ACCACAATTCTGTCTTTCACGG -3' (start position 4126, within the second exon); for PR: forward primer, 5'-TCAGTGGGCAGATGC TGTATTT-3' and reverse primer, 5'-GCCACATGGTAAGGCATAATGA-3' (70); for cathepsin D: forward primer, 5'-

455 GTACATGATCCCCTGTGAGAAGGT-3'; reverse primer, 5'-
GGGACAGCTTGTAGCCTTTGC-3' (71); and for β -actin: forward primer, 5'-
TGCGTGACATTAAGGAGAAG-3' and reverse primer, 5'-GCTCGTAGCT CTTCTCCA-3'.
Q-PCR was performed in 96-well optical plates (Bio-Rad, Hercules, CA) using an iCycler
system (Bio-Rad) for 40 cycles (94°C for 10 sec, 60°C for 40 sec), following an initial 3 min
460 denaturation at 94°C. The relative concentration of RNA was calculated using the $\Delta\Delta C_t$ method
according to Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin) with
 β -actin mRNA as an internal control. Results were expressed as relative RNA levels
standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1.

465 REFERENCE

1. McKenna NJ, O'Malley BW 2001 Nuclear receptors, coregulators, ligands, and selective receptor modulators: making sense of the patchwork quilt. *Ann N Y Acad Sci* 949:3-5
2. Webb P, Lopez GN, Greene GL, Baxter JD, Kushner PJ 1992 The limits of the cellular capacity to mediate an estrogen response. *Mol Endocrinol* 6:157-67
- 470 3. Klinge CM 2000 Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 65:227-51
4. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Mol Endocrinol* 2:1157-62
- 475 5. Pink JJ, Jordan VC 1996 Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56:2321-30
6. Nephew KP, Long X, Osborne E, Burke KA, Ahluwalia A, Bigsby RM 2000 Effect of estradiol on estrogen receptor expression in rat uterine cell types. *Biol Reprod* 62:168-77
7. Lonard DM, Nawaz Z, Smith CL, O'Malley BW 2000 The 26S proteasome is required
480 for estrogen receptor- α and coactivator turnover and for efficient estrogen receptor- α transactivation. *Mol Cell* 5:939-48
8. Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW 1999 Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci U S A* 96:1858-62
9. Alarid ET, Bakopoulos N, Solodin N 1999 Proteasome-mediated proteolysis of estrogen
485 receptor: a novel component in autologous down-regulation. *Mol Endocrinol* 13:1522-34
10. El Khissiin A, Leclercq G 1999 Implication of proteasome in estrogen receptor degradation. *FEBS Lett* 448:160-6

11. Wijayaratne AL, McDonnell DP 2001 The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 276:35684-92
12. Muratani M, Tansey WP 2003 How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4:192-201
13. Conaway RC, Brower CS, Conaway JW 2002 Emerging roles of ubiquitin in transcription regulation. Science 296:1254-8
14. Tansey WP 2001 Transcriptional activation: risky business. Genes Dev 15:1045-50
15. Salghetti SE, Muratani M, Wijnen H, Futcher B, Tansey WP 2000 Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. Proc Natl Acad Sci U S A 97:3118-23
16. Salghetti SE, Caudy AA, Chenoweth JG, Tansey WP 2001 Regulation of transcriptional activation domain function by ubiquitin. Science 293:1651-3
17. Wallace AD, Cidlowski JA 2001 Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem 276:42714-21
18. Pollenz RS 2002 The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. Chem Biol Interact 141:41-61
19. Blanquart C, Barbier O, Fruchart JC, Staels B, Glineur C 2002 Peroxisome proliferator-activated receptor alpha (PPARalpha) turnover by the ubiquitin-proteasome system controls the ligand-induced expression level of its target genes. J Biol Chem 277:37254-9

20. Boudjelal M, Voorhees JJ, Fisher GJ 2002 Retinoid signaling is attenuated by
510 proteasome-mediated degradation of retinoid receptors in human keratinocyte HaCaT
cells. *Exp Cell Res* 274:130-7
21. Li XY, Boudjelal M, Xiao JH, Peng ZH, Asuru A, Kang S, Fisher GJ, Voorhees JJ 1999
1,25-Dihydroxyvitamin D3 increases nuclear vitamin D3 receptors by blocking
ubiquitin/proteasome-mediated degradation in human skin. *Mol Endocrinol* 13:1686-94
- 515 22. Kang Z, Pirskanen A, Janne OA, Palvimo JJ 2002 Involvement of proteasome in the
dynamic assembly of the androgen receptor transcription complex. *J Biol Chem*
277:48366-71
23. Lin HK, Altuwaijri S, Lin WJ, Kan PY, Collins LL, Chang C 2002 Proteasome activity is
required for androgen receptor transcriptional activity via regulation of androgen receptor
520 nuclear translocation and interaction with coregulators in prostate cancer cells. *J Biol*
Chem 277:36570-6
24. Stavreva DA, Muller WG, Hager GL, Smith CL, McNally JG 2004 Rapid glucocorticoid
receptor exchange at a promoter is coupled to transcription and regulated by chaperones
and proteasomes. *Mol Cell Biol* 24:2682-97
- 525 25. Ciechanover A, Orian A, Schwartz AL 2000 Ubiquitin-mediated proteolysis: biological
regulation via destruction. *Bioessays* 22:442-51
26. Molinari E, Gilman M, Natesan S 1999 Proteasome-mediated degradation of
transcriptional activators correlates with activation domain potency in vivo. *Embo J*
18:6439-47

- 530 27. Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai MJ, O'Malley BW 1999
The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear
hormone receptor superfamily. *Mol Cell Biol* 19:1182-9
28. Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H, Nakatani Y,
Livingston DM 2003 Polyubiquitination of p53 by a ubiquitin ligase activity of p300.
535 *Science* 300:342-4
29. Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R, Brown M
1996 p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad
Sci U S A* 93:11540-5
30. Ruffner H, Joazeiro CA, Hemmati D, Hunter T, Verma IM 2001 Cancer-predisposing
540 mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity
and protection from radiation hypersensitivity. *Proc Natl Acad Sci U S A* 98:5134-9.
31. Fan S, Wang J, Yuan R, Ma Y, Meng Q, Erdos MR, Pestell RG, Yuan F, Auburn KJ,
Goldberg ID, Rosen EM 1999 BRCA1 inhibition of estrogen receptor signaling in
transfected cells. *Science* 284:1354-6
- 545 32. Fraser RA, Rossignol M, Heard DJ, Egly JM, Chambon P 1997 SUG1, a putative
transcriptional mediator and subunit of the PA700 proteasome regulatory complex, is a
DNA helicase. *J Biol Chem* 272:7122-6.
33. Saji S, Okumura N, Eguchi H, Nakashima S, Suzuki A, Toi M, Nozawa Y, Hayashi S
2001 MDM2 enhances the function of estrogen receptor alpha in human breast cancer
550 cells. *Biochem Biophys Res Commun* 281:259-65

34. Masuyama H, Hiramatsu Y 2004 Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors. *J Biol Chem* 279:12020-6
35. Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F 2003 Cyclic, Proteasome-Mediated Turnover of Unliganded and Liganded ERalpha on Responsive Promoters Is an Integral Feature of Estrogen Signaling. *Mol Cell* 11:695-707
36. Deroo BJ, Archer TK 2002 Proteasome inhibitors reduce luciferase and beta-galactosidase activity in tissue culture cells. *J Biol Chem* 277:20120-3
37. Laios I, Journe F, Laurent G, Nonclercq D, Toillon RA, Seo HS, Leclercq G 2003 Mechanisms governing the accumulation of estrogen receptor alpha in MCF-7 breast cancer cells treated with hydroxytamoxifen and related antiestrogens. *J Steroid Biochem Mol Biol* 87:207-21
38. Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS 2004 Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res* 64:1522-33
39. Alarid ET, Preisler-Mashek MT, Solodin NM 2003 Thyroid Hormone Is an Inhibitor of Estrogen-Induced Degradation of Estrogen Receptor-alpha Protein: Estrogen-Dependent Proteolysis Is Not Essential for Receptor Transactivation Function in the Pituitary. *Endocrinology* 144:3469-76
40. Schreihof DA, Resnick EM, Lin VY, Shupnik MA 2001 Ligand-independent activation of pituitary ER: dependence on PKA-stimulated pathways. *Endocrinology* 142:3361-8

41. Wada H, Yeh ET, Kamitani T 2000 A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. *J Biol Chem* 275:17008-15
- 575 42. Fan M, Bigsby RM, Nephew KP 2003 The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the antiproliferative activity of ICI 182,780 in ERalpha-positive breast cancer cells. *Mol Endocrinol* 17:356-65
43. Bloom J, Amador V, Bartolini F, DeMartino G, Pagano M 2003 Proteasome-mediated
580 degradation of p21 via N-terminal ubiquitinylation. *Cell* 115:71-82
44. Thompson JF, Hayes LS, Lloyd DB 1991 Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* 103:171-7
45. Berry M, Nunez AM, Chambon P 1989 Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc Natl Acad Sci U S A* 86:1218-22
- 585 46. Brown AM, Jeltsch JM, Roberts M, Chambon P 1984 Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *Proc Natl Acad Sci U S A* 81:6344-8
47. Lipson KE, Baserga R 1989 Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific
590 probe. *Proc Natl Acad Sci U S A* 86:9774-7
48. Tian Y, Ke S, Thomas T, Meeker RJ, Gallo MA 1998 Transcriptional suppression of estrogen receptor gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *J Steroid Biochem Mol Biol* 67:17-24

49. Elferink CJ, Reiners JJ, Jr. 1996 Quantitative RT-PCR on CYP1A1 heterogeneous
595 nuclear RNA: a surrogate for the in vitro transcription run-on assay. *Biotechniques*
20:470-7
50. Delany AM 2001 Measuring transcription of metalloproteinase genes. Nuclear run-off
assay vs analysis of hnRNA. *Methods Mol Biol* 151:321-33
51. Anzai Y, Holinka CF, Kuramoto H, Gurside E 1989 Stimulatory effects of 4-
600 hydroxytamoxifen on proliferation of human endometrial adenocarcinoma cells
(Ishikawa line). *Cancer Res* 49:2362-5
52. Jamil A, Croxtall JD, White JO 1991 The effect of anti-oestrogens on cell growth and
progesterone receptor concentration in human endometrial cancer cells (Ishikawa). *J Mol*
Endocrinol 6:215-21
- 605 53. Shang Y, Brown M 2002 Molecular determinants for the tissue specificity of SERMs.
Science 295:2465-8
54. Barkhem T, Haldosen LA, Gustafsson JA, Nilsson S 2002 pS2 Gene expression in
HepG2 cells: complex regulation through crosstalk between the estrogen receptor alpha,
an estrogen-responsive element, and the activator protein 1 response element. *Mol*
610 *Pharmacol* 61:1273-83
55. Lee YJ, Gorski J 1996 Estrogen-induced transcription of the progesterone receptor gene
does not parallel estrogen receptor occupancy. *Proc Natl Acad Sci U S A* 93:15180-4
56. Makino Y, Yogosawa S, Kayukawa K, Coin F, Egly JM, Wang Z, Roeder RG,
Yamamoto K, Muramatsu M, Tamura T 1999 TATA-Binding protein-interacting protein
615 120, TIP120, stimulates three classes of eukaryotic transcription via a unique mechanism.
Mol Cell Biol 19:7951-60

57. Russell SJ, Johnston SA 2001 Evidence that proteolysis of Gal4 cannot explain the transcriptional effects of proteasome ATPase mutations. *J Biol Chem* 276:9825-31
58. Ferdous A, Gonzalez F, Sun L, Kodadek T, Johnston SA 2001 The 19S regulatory
620 particle of the proteasome is required for efficient transcription elongation by RNA
polymerase II. *Mol Cell* 7:981-91
59. Gonzalez F, Delahodde A, Kodadek T, Johnston SA 2002 Recruitment of a 19S
proteasome subcomplex to an activated promoter. *Science* 296:548-50
60. Ferdous A, Kodadek T, Johnston SA 2002 A nonproteolytic function of the 19S
625 regulatory subunit of the 26S proteasome is required for efficient activated transcription
by human RNA polymerase II. *Biochemistry* 41:12798-805
61. Yan F, Gao X, Lonard DM, Nawaz Z 2003 Specific ubiquitin-conjugating enzymes
promote degradation of specific nuclear receptor coactivators. *Mol Endocrinol* 17:1315-
31
- 630 62. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y 1987 Proliferation, hormonal
responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown
in the short-term and long-term absence of estrogens. *Cancer Res* 47:4355-60
63. Panno ML, Salerno M, Pezzi V, Sisci D, Maggiolini M, Mauro L, Morrone EG, Ando S
1996 Effect of oestradiol and insulin on the proliferative pattern and on oestrogen and
635 progesterone receptor contents in MCF-7 cells. *J Cancer Res Clin Oncol* 122:745-9
64. Clarke RB, Howell A, Potten CS, Anderson E 1997 Dissociation between steroid
receptor expression and cell proliferation in the human breast. *Cancer Res* 57:4987-91

65. Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, Sibson DR, Sloane
JP 1999 Estrogen receptor-positive proliferating cells in the normal and precancerous
640 breast. *Am J Pathol* 155:1811-5
66. Shoker BS, Jarvis C, Clarke RB, Anderson E, Munro C, Davies MP, Sibson DR, Sloane
JP 2000 Abnormal regulation of the oestrogen receptor in benign breast lesions. *J Clin
Pathol* 53:778-83
67. Fan M, Long X, Bailey JA, Reed CA, Osborne E, Gize EA, Kirk EA, Bigsby RM,
645 Nephew KP 2002 The activating enzyme of NEDD8 inhibits steroid receptor function.
Mol Endocrinol 16:315-30
68. Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai MJ, O'Malley BW 1989 Specific
binding of estrogen receptor to the estrogen response element. *Mol Cell Biol* 9:43-9
69. Rajendran RR, Nye AC, Frasor J, Balsara RD, Martini PG, Katzenellenbogen BS 2003
650 Regulation of nuclear receptor transcriptional activity by a novel DEAD box RNA
helicase (DP97). *J Biol Chem* 278:4628-38
70. Aerts JL, Christiaens MR, Vandekerckhove P 2002 Evaluation of progesterone receptor
expression in eosinophils using real-time quantitative PCR. *Biochim Biophys Acta*
1571:167-72
- 655 71. Augereau P, Miralles F, Cavailles V, Gaudet C, Parker M, Rochefort H 1994
Characterization of the proximal estrogen-responsive element of human cathepsin D
gene. *Mol Endocrinol* 8:693-703

FIGURE LEGENDS

660 **Fig. 1.** Proteasome inhibition enhances E2-induced CAT reporter gene expression in HeLa cells transfected with ER α .

A. Effect of proteasome inhibition by MG132 on expression of reporter enzymes from constitutively active promoters. HeLa cells (*left panel*) were plated on 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 3 days. The cells were
665 transfected with 100 ng RSV-CAT, 100 ng SV40-Luc or 5 ng pCMV- β -gal using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO) or 1 μ M MG132 for 24 h. Similarly, MCF7 cells (*right panel*) were plated at a density of 1.2×10^5 cells/well, transfected with 250 ng RSV-CAT, 250 ng SV40-Luc or 10 ng pCMV- β -gal
670 and then treated with DMSO or MG132 for 24 h. Reporter enzyme activities were normalized against total cellular protein and expressed as the mean \pm SD from three independent experiments, each in triplicate. *B. Effect of MG132 on ER α -mediated CAT expression.* HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 100 ng ERE-vit-CAT and the indicated amount of pSG5-ER α using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture
675 was removed and cells were placed in hormone-free medium for 24 h. Transfected cells were treated with DMSO or MG132 (1 μ M) for 1 h and then treated with 10 nM E2 for 24 h. CAT activity was determined using the colorimetric CAT ELISA kit and normalized against total cellular protein. CAT activity is expressed as the mean \pm SD of three independent experiments, each performed in triplicate. Fold increases in ERE-CAT in the presence of E2 \pm MG132 are
680 presented in the table. *C. Effect of MG132 on E2-induced down-regulation of ER α .* HeLa cells

were plated in 60-mm dishes at a density of 3×10^5 cells/dish and cultured in hormone-free medium for 2 days. Cells were transfected with 100 ng pSG5-ER α using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed, and cells were
685 placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1 μ M) for 1 h and then treated with 10 nM E2 for 8 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER α antibody (Chemicon). GAPDH was used as a loading control.

690 **Fig. 2.** Effect of MG132 on E2 dose-dependent induction of reporter gene expression in HeLa cells.

HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 100 ng ERE-vit-CAT and 0.3 ng (A) or 1 ng (B) of pSG5-ER α using LipofectAMINE Plus Reagent. Five hours later, the
695 DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1 μ M) for 1 h and then treated with the indicated concentration of E2 for 24 h. CAT activities were normalized against total cellular protein and expressed as mean \pm SD of three independent experiments, each performed in triplicate. EC50 range was calculated with a 95% confidence.

700

Fig. 3. Effect of blocking ER α turnover on time-dependent induction of reporter gene expression by E2 in HeLa cells.

A. Effect of MG132 on E2-induced expression of reporter gene. HeLa cells were plated in 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 2 days.

705 The cells were transfected with 250 ng ERE-pS2-Luc and 1 ng of pSG5-ER α using
 LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was
 removed and cells were placed in hormone-free medium for 24 h. The transfected cells were
 treated with DMSO or MG132 (5 μ M) for 1 h and then treated with 10 nM E2 for indicated time
 period. Luciferase activity was determined using the Luciferase Assay System, normalized
 710 against total cellular protein. *B. Effect of MG132 on SV40-Luc expression.* HeLa cells were
 transfected with 100 ng SV40-Luc. Five hours later, the DNA/LipofectAMINE mixture was
 removed and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO)
 or MG132 (5 μ M) for the indicated time period. Luciferase activity was determined and
 normalized against total cellular protein. *C. Normalized ERE-Luc activities.* ER α -mediated
 715 luciferase activity in the presence of MG132 was normalized to luciferase activity from the
 SV40-Luc construct (Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc
 activity in the presence of MG132 \times [SV40-Luc activity/SV40-Luc activity in the presence of
 MG132]). *D. Effect of overexpressing Ubc12C111S on E2-induced reporter gene expression.*
 HeLa cells were transfected with 250 ng ERE-pS2-Luc, 1 ng of pSG5-ER α , along with 100 ng
 720 pcDNA or pcDNA-Ubc12C111S and treated with 10 nM E2 for the indicated period of time. Luc
 activities were normalized against total cellular protein. *E. Effect of overexpressing*
Ubc12C111S on SV40-Luc expression. HeLa cells were transfected with 100 ng SV40-Luc,
 along with 100 ng pcDNA-Ubc12C111S or control vector pcDNA. Five hours later, the
 DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for
 725 the indicated time period. Luc activities were normalized against total cellular protein. For all
 assays, Luc activities are expressed as mean \pm SD from three independent experiments, each
 performed in triplicate.

Fig. 4. Ubiquitin mutant blocks ER degradation and sustained E2-induced gene expression.

730 *A. Overexpression of UbK0 blocks E2-induced ER α degradation.* HeLa cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish and cultured in hormone-free medium for 2 days. The cells were transfected with 150 ng pSG5-ER α , along with 150 ng pcDNA-Ub or pCS2-UbK0 using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h prior to treatment with DMSO
735 or 10 nM E2 for 8 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER α antibody. The coomassie stained SDS-PAGE gels show that equal amounts of cell lysates were loaded. *B. Effect of UbK0 on ER α -mediated luciferase expression.* HeLa cells stably transfected with ER α were plated in 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 250 ng ERE-pS2-
740 Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0 as indicated, using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h prior to treatment with DMSO or 10 nM E2 for the indicated time period. *C. Effect of UbK0 on luciferase expression from SV40-Luc.* HeLa cells stably transfected with ER α were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-
745 Ub or pCS2-UbK0. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for indicated time period. Luciferase activity was normalized against total cellular protein and expressed as the mean \pm SD from three independent experiments, each performed in triplicate.

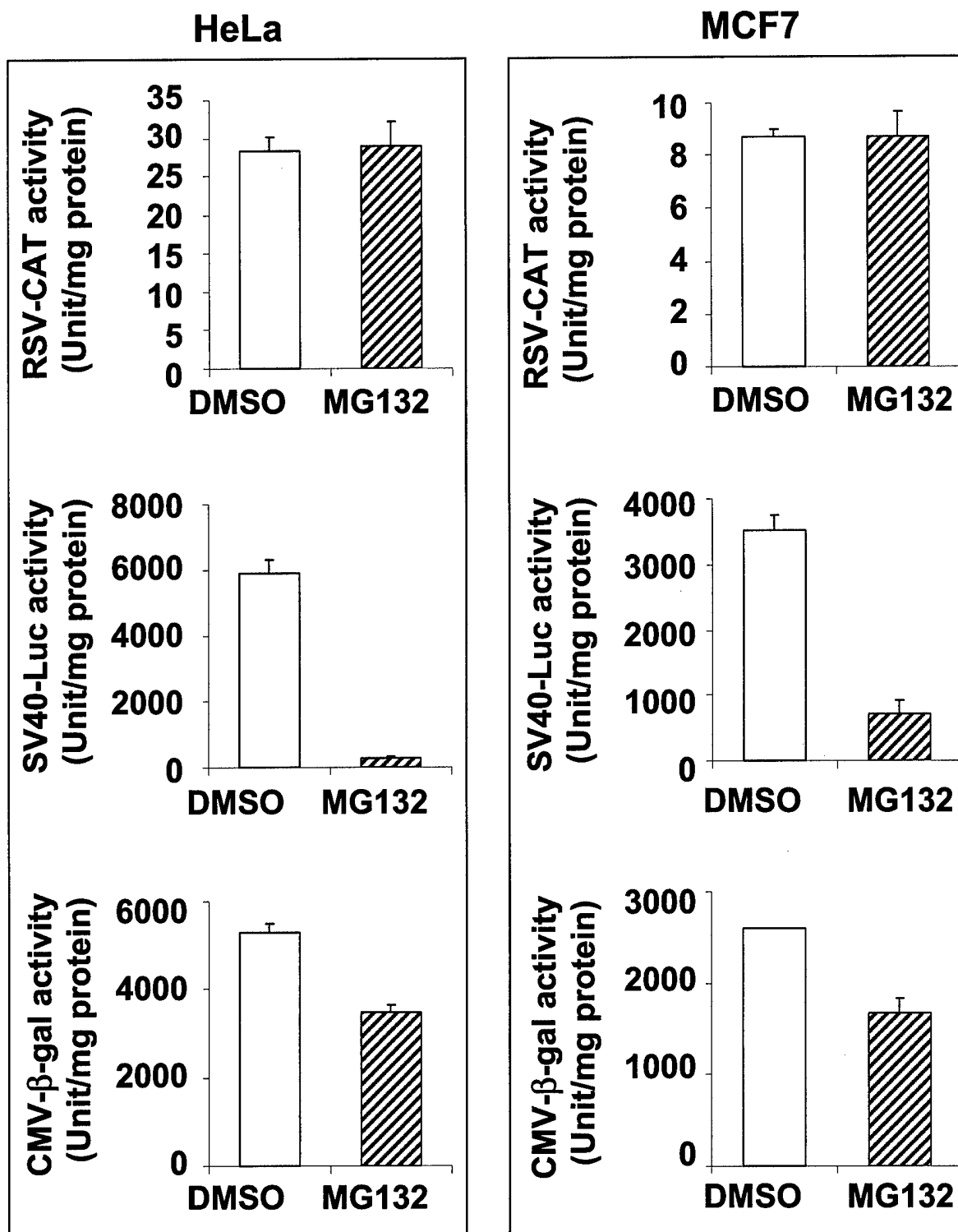
750 **Fig. 5.** Effects of MG132 on ER α -mediated transcription of endogenous target genes in MCF7 cells.

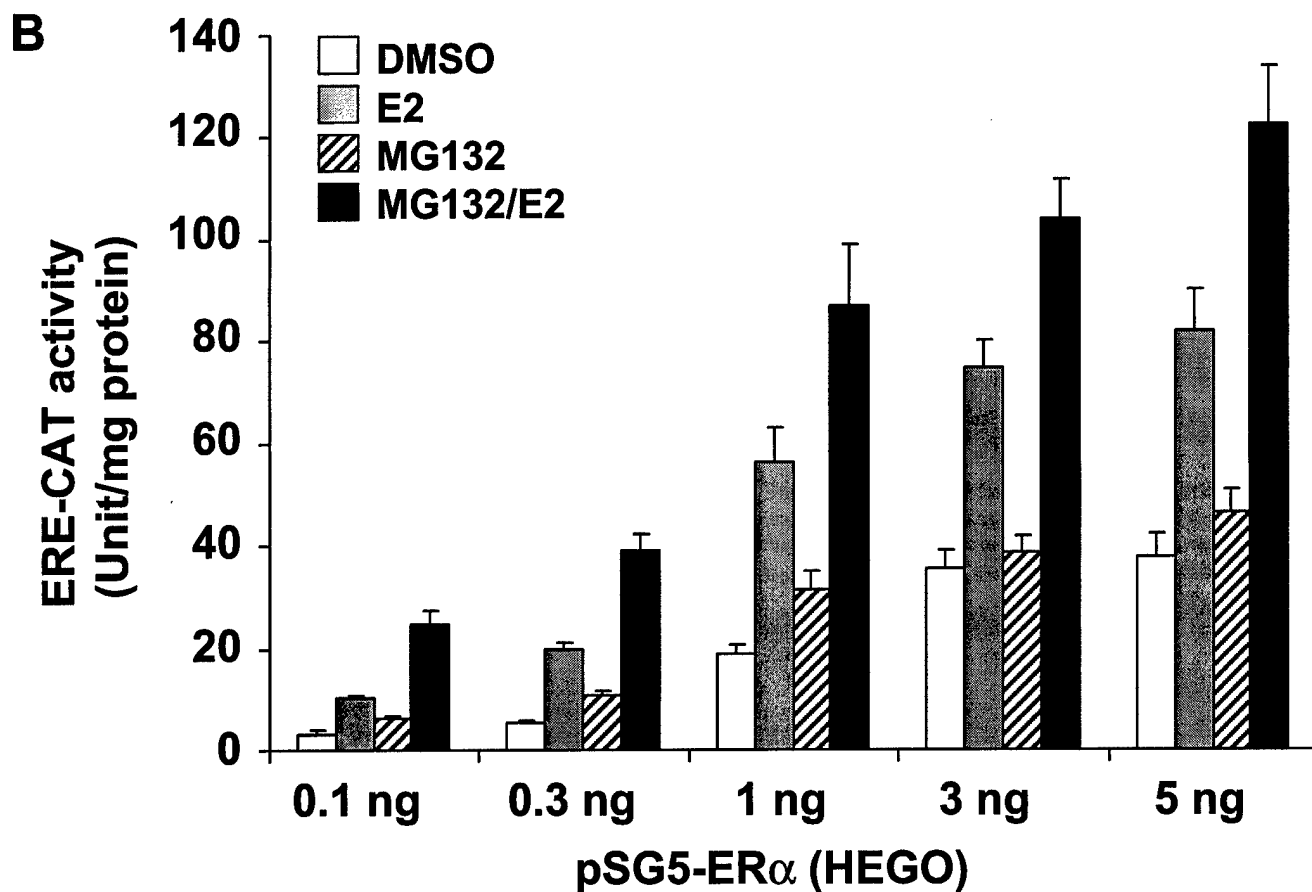
MCF7 cells were plated at a density of 3×10^6 per 10-cm dish and allowed to grow in hormone-free medium for 3 days. The cells were pretreated with MG132 (5 μ M) for 1 h and then treated with 10 nM E2 for the indicated time periods. Total RNA was prepared and subjected to Q-PCR
755 analysis to determine the expression levels of pS2 hnRNA (A), pS2 mRNA (B), cathepsin D mRNA (C) and PR mRNA (D). For all Q-PCR assays, the relative levels of mRNA were normalized with β -actin mRNA and standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1. The results were expressed as mean \pm SD from two independent experiments, each in duplicate. To determine the effect of MG132 on E2-induced
760 ER degradation, MCF7 cells were treated as in A and subjected to whole cell lysate preparation and immunoblotting with an anti-ER antibody (E). GAPDH was used as a loading control.

Fig. 6. Uncoupling of 4-OHT induced ER α activation and ER α degradation.

765 *A. 4-OHT stimulates ER α -mediated gene expression in Ishikawa cells.* Ishikawa cells stably transfected with ER α were plated in 12-well dishes at a density of 1×10^5 cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 250 ng C3T1-Luc, along with 100 ng pcDNA or pcDNA-SRC1 using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for
770 24 h prior to treatment with 10 nM E2 or 1 μ M 4-OHT for 16 h. Luciferase activity was normalized against total cellular protein and expressed as mean \pm SD from three independent experiments, each performed in triplicate. *B. Effect of 4-OHT on ER α protein level.* Ishikawa cells stably transfected with ER α were plated in 60-mm dishes at a density of 3×10^5 cells/dish

and cultured in hormone-free medium for 3 days prior to treatment with 10 nM E2 or 1 μ M 4-
775 OHT for 16 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using
an anti-ER antibody. GAPDH was used as a loading control.

A**Fig 1A**



The fold increases of ERE-CAT (relative to DMSO)

| | pSG5-ER α | | | | |
|----------|------------------|--------|------|------|------|
| | 0.1 ng | 0.3 ng | 1 ng | 3 ng | 5 ng |
| DMSO | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| E2 | 3.10 | 3.70 | 3.00 | 2.09 | 2.17 |
| MG132 | 1.82 | 2.01 | 1.68 | 1.08 | 1.22 |
| MG132/E2 | 7.40 | 7.31 | 4.63 | 2.91 | 3.23 |

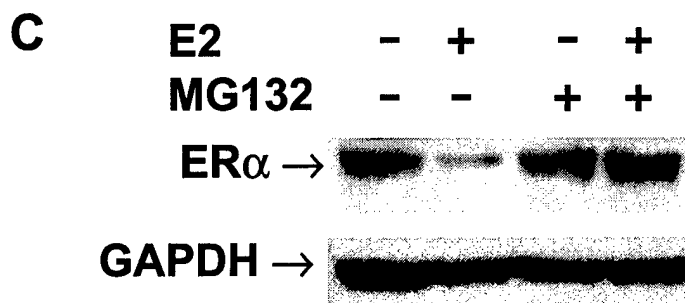


Fig 1B,C

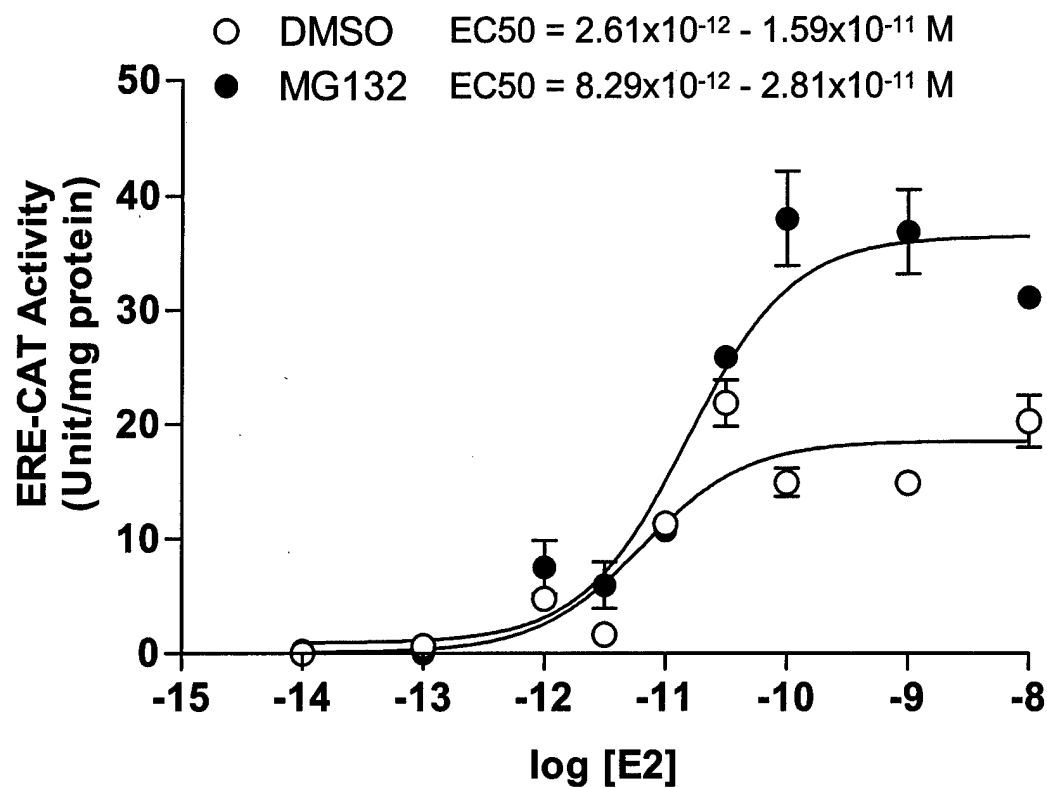
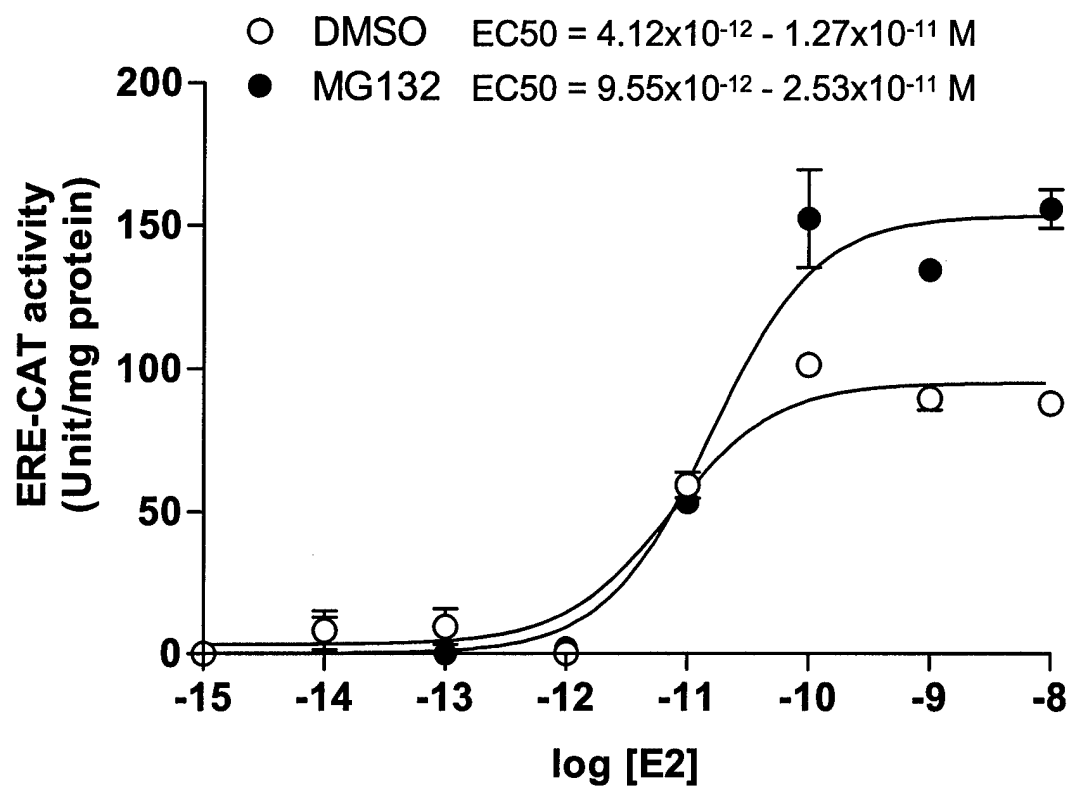
A**B**

Fig 2

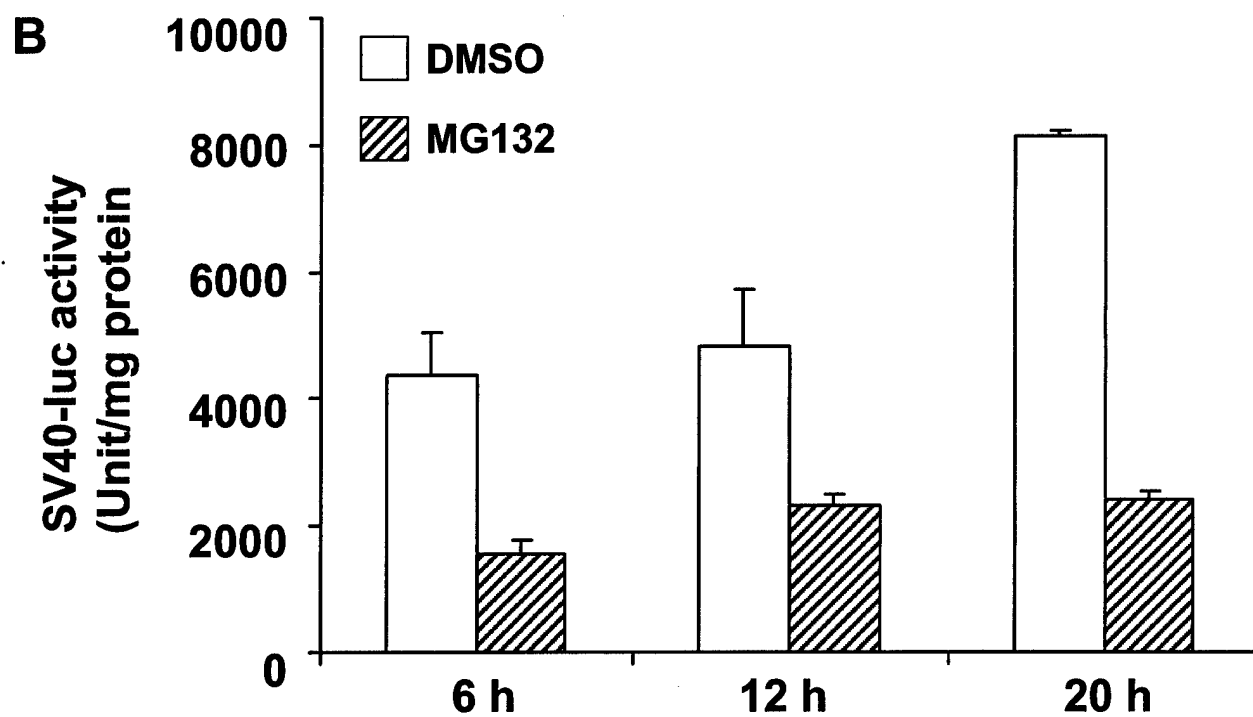
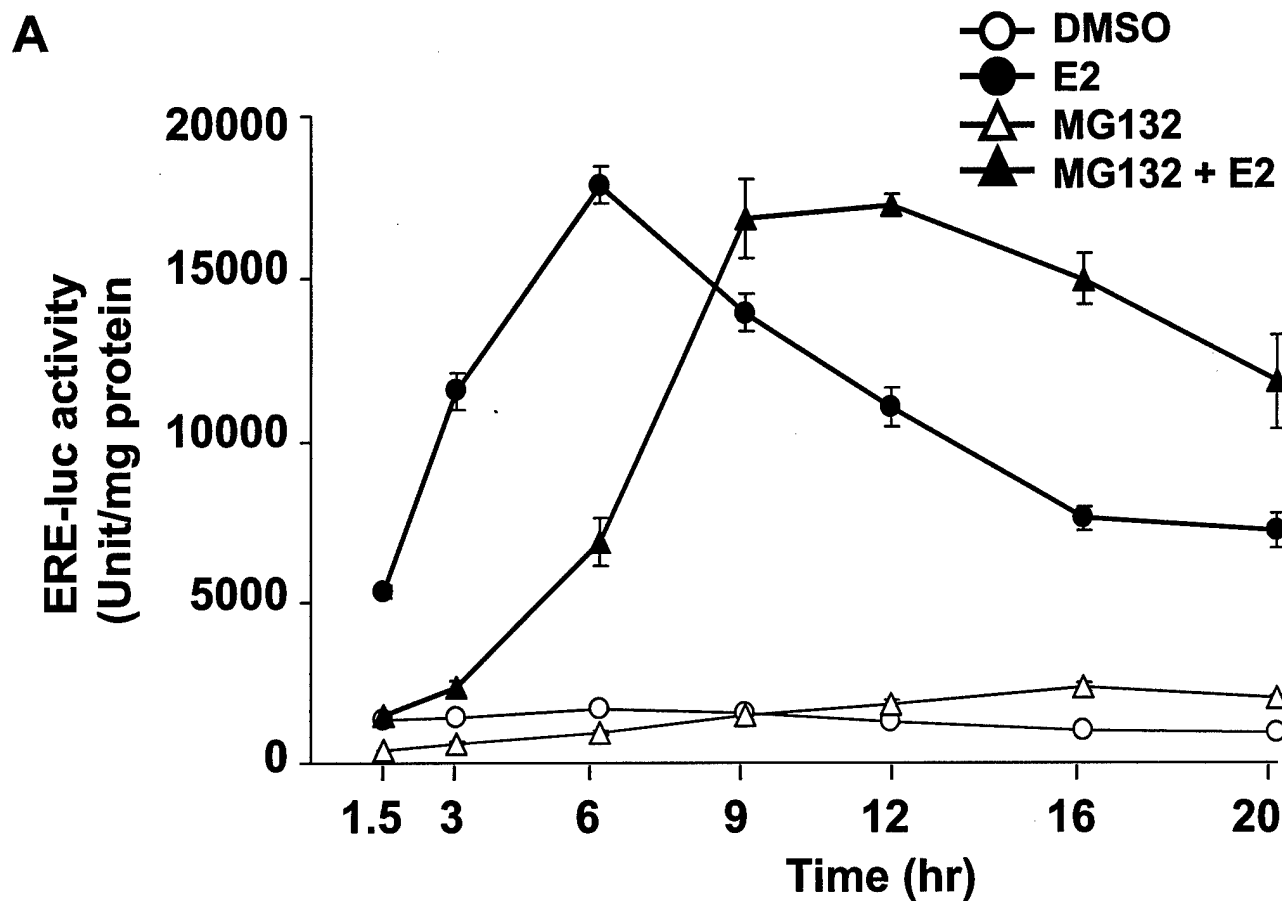


Fig 3A,B

C

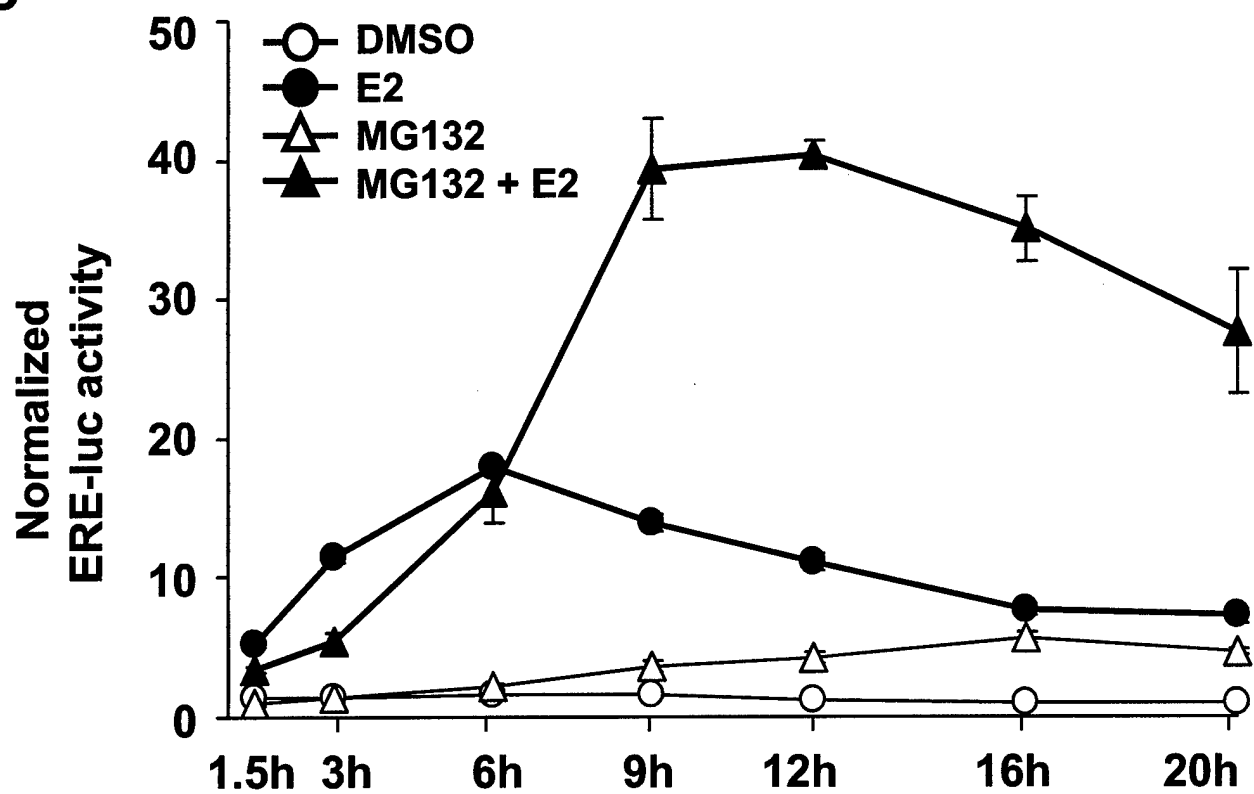


Fig 3C

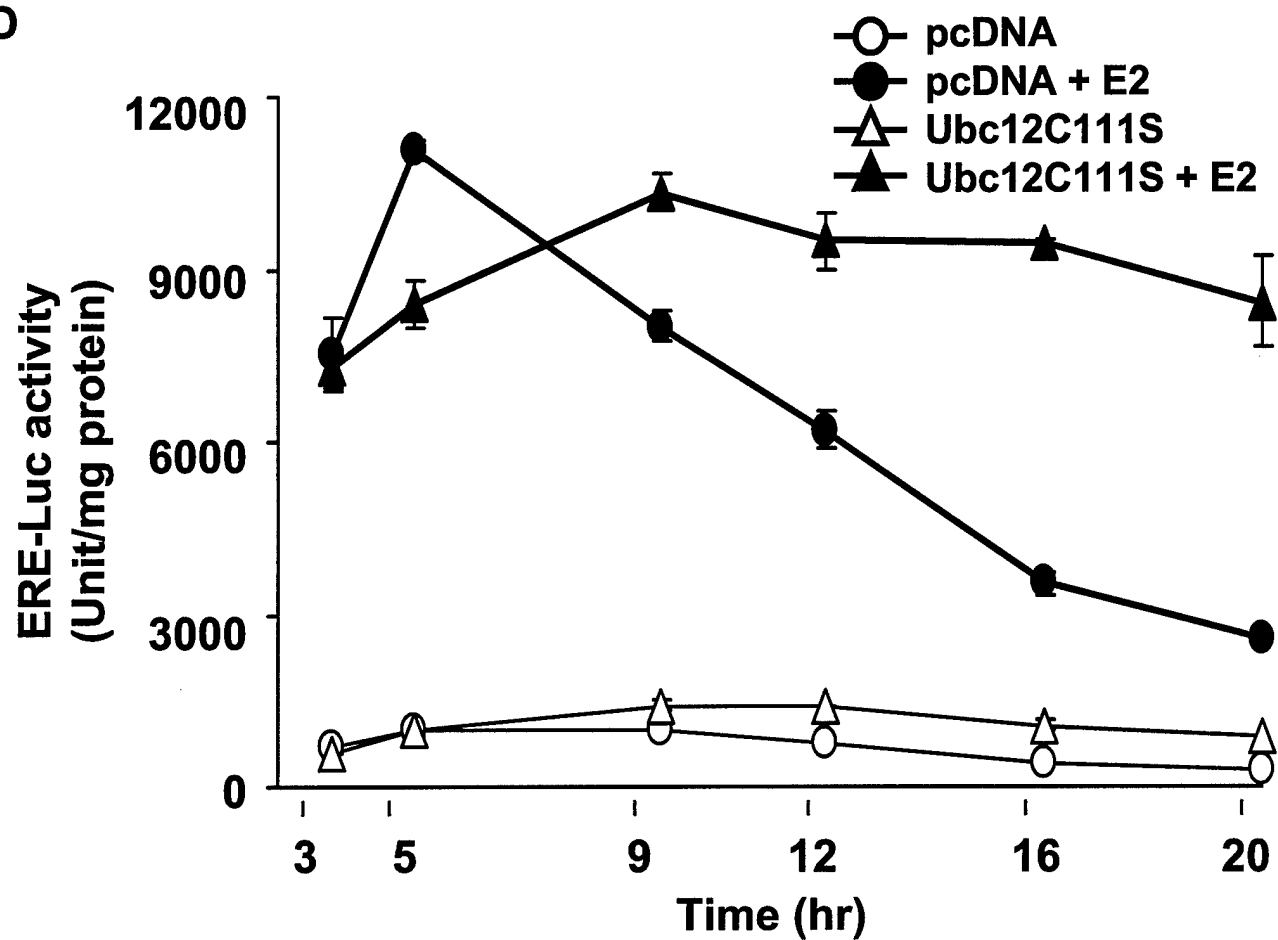
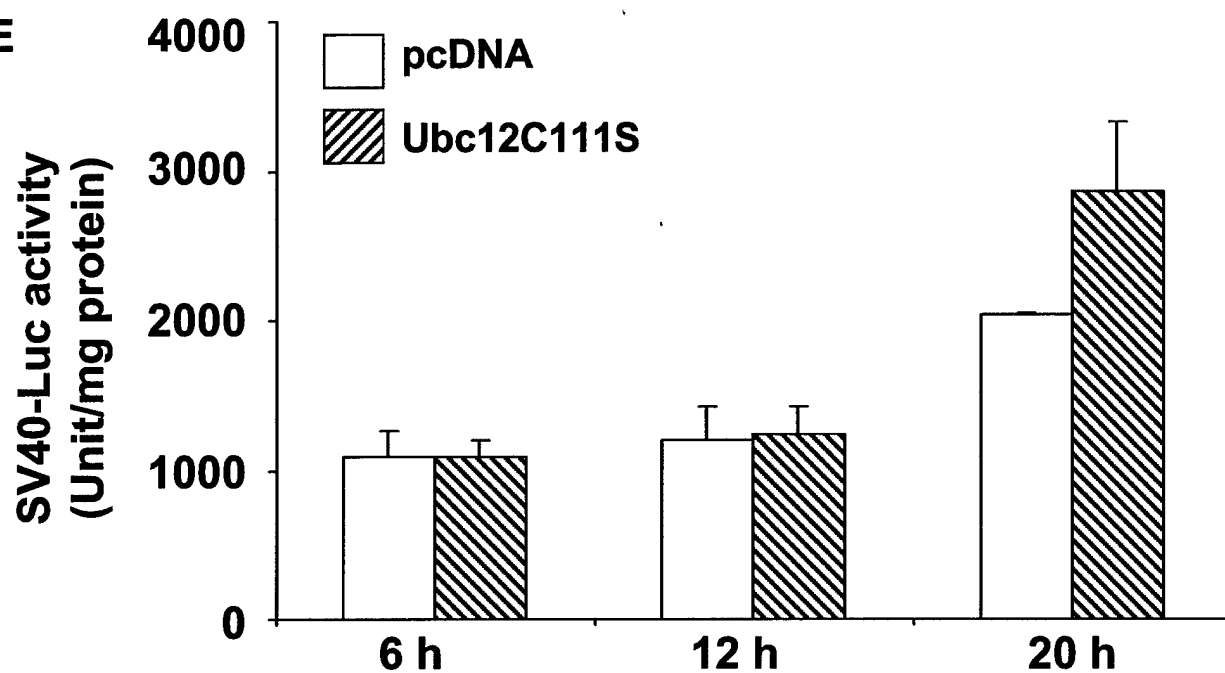
D**E**

Fig 3D,E

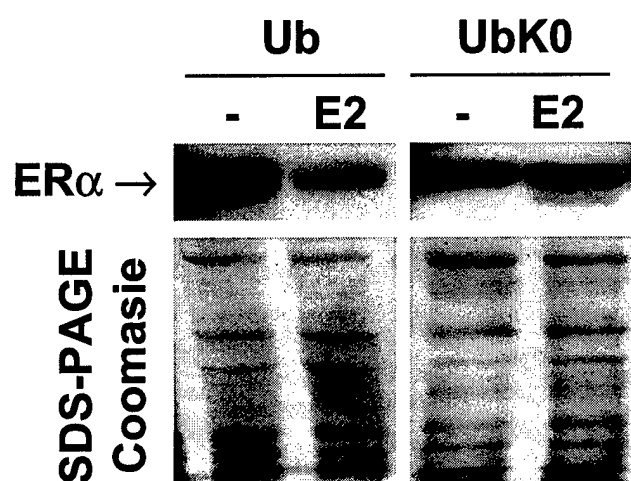
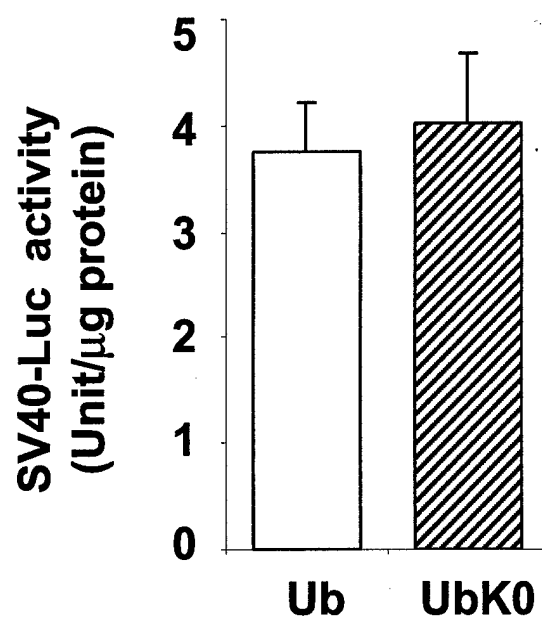
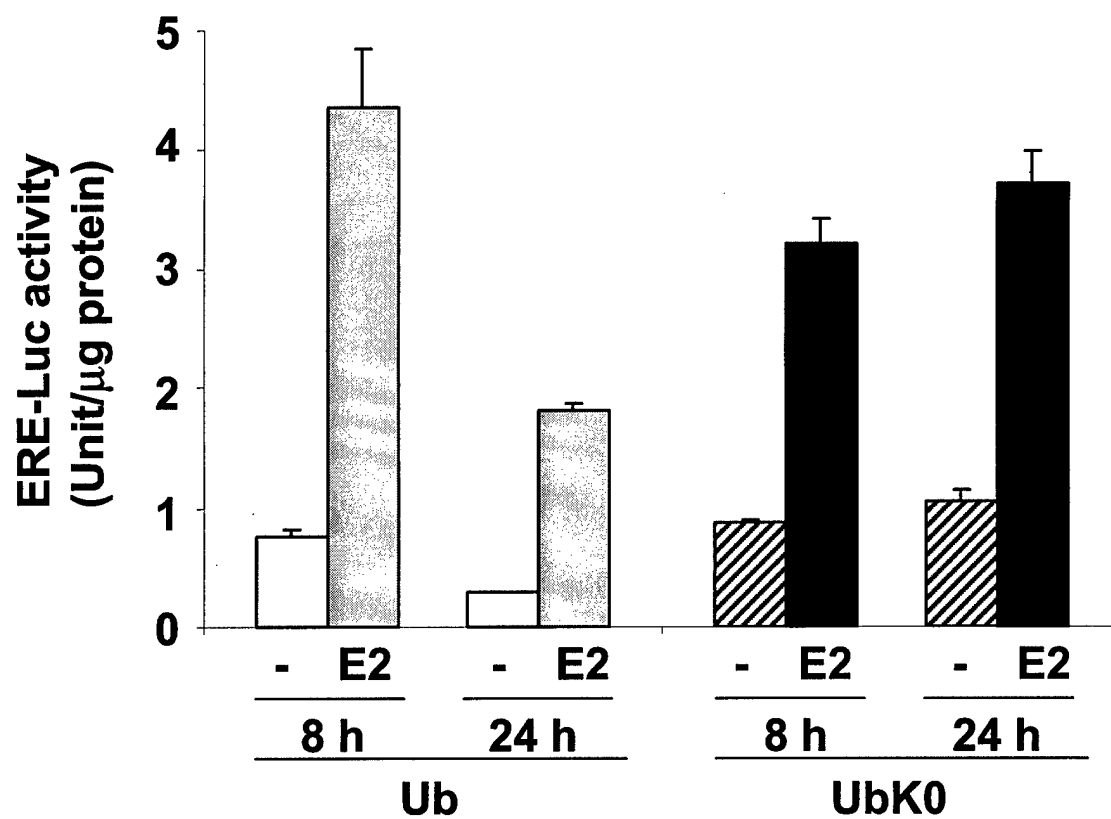
A**C****B**

Fig 4

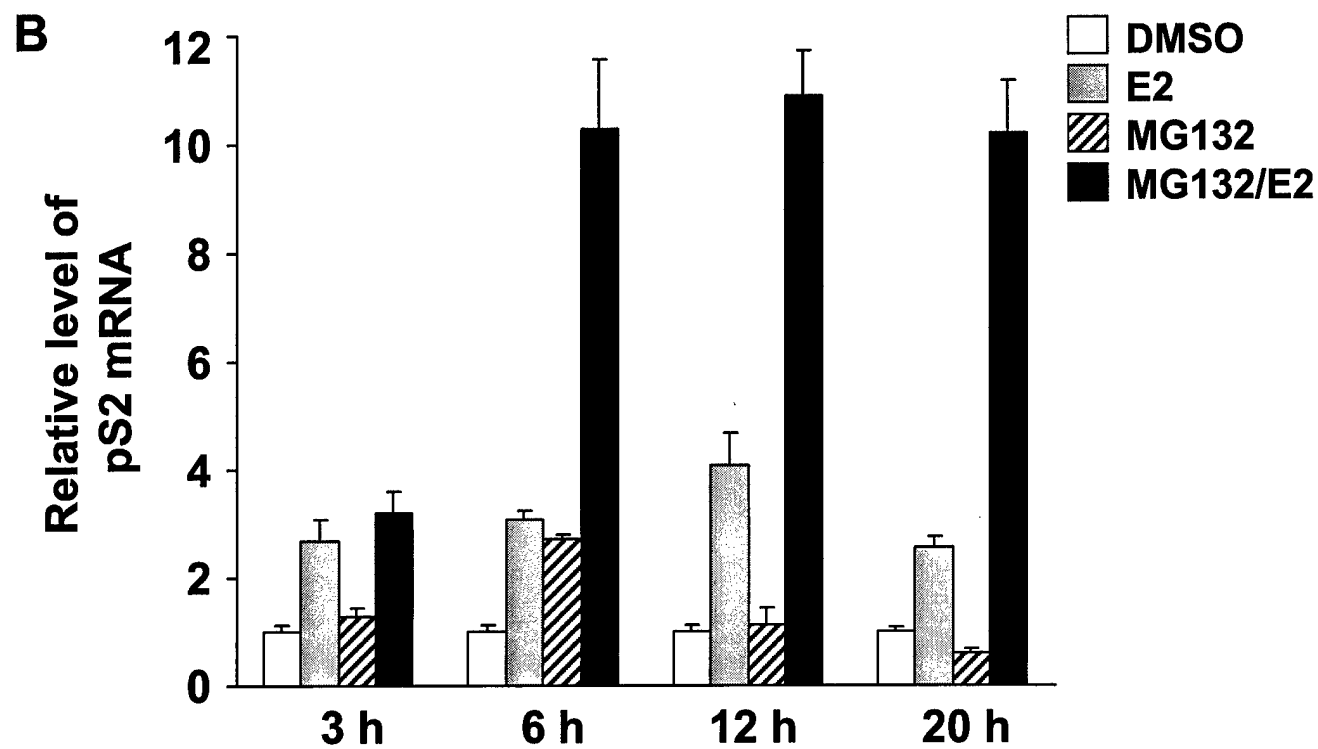
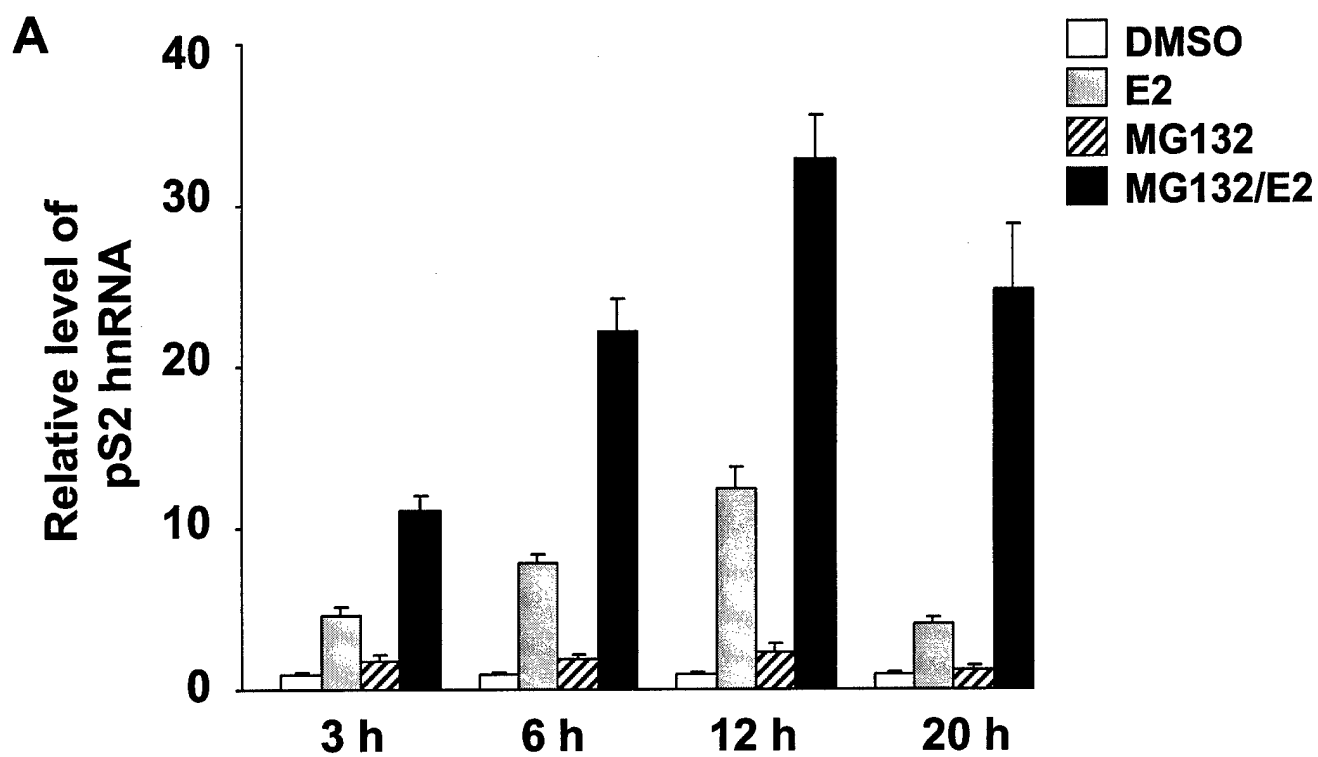


Fig 5A,B

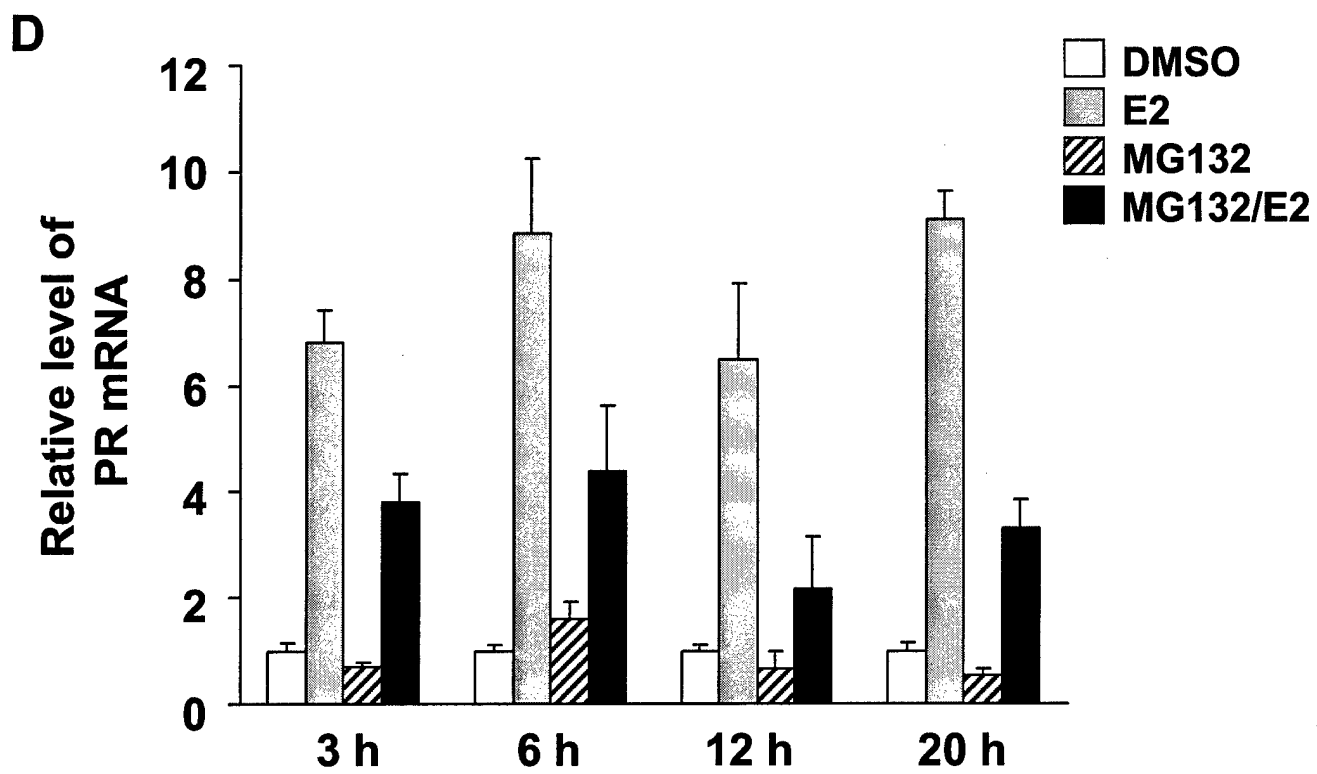
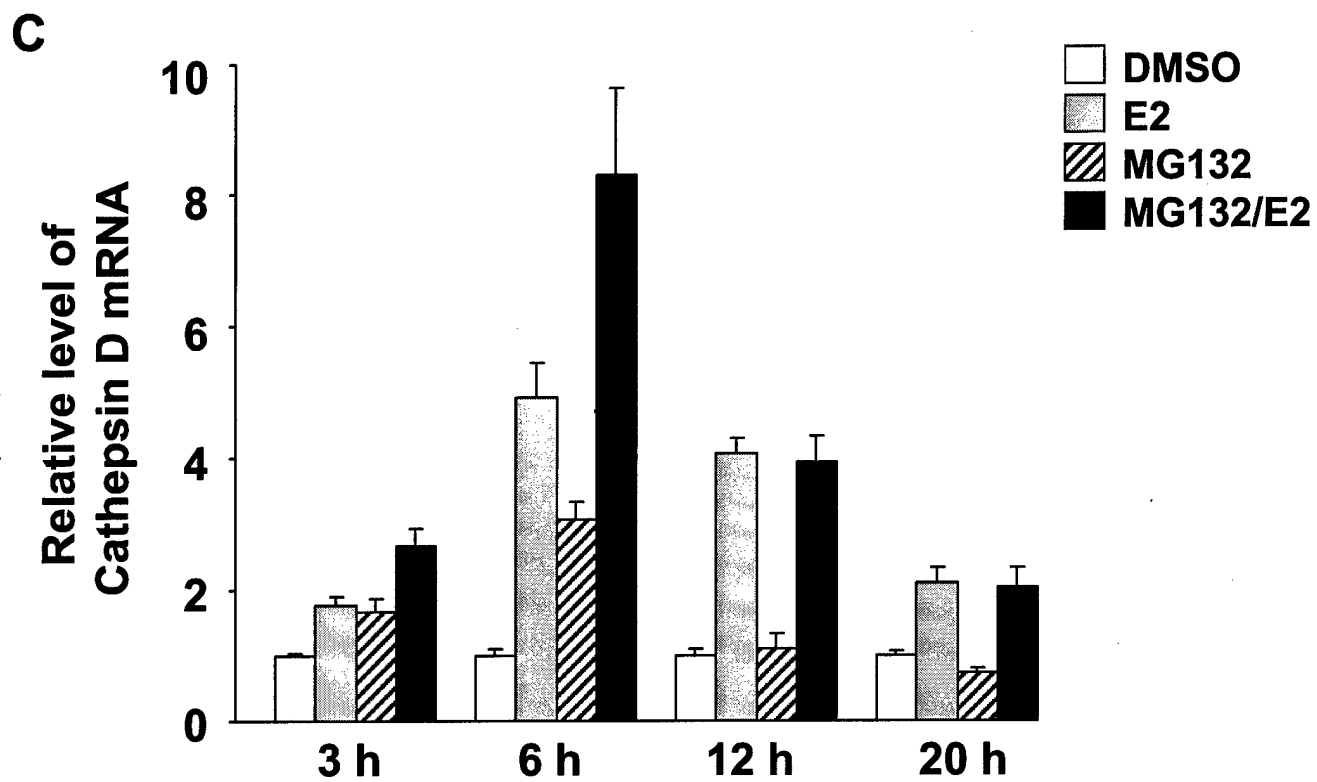


Fig 5C,D

E

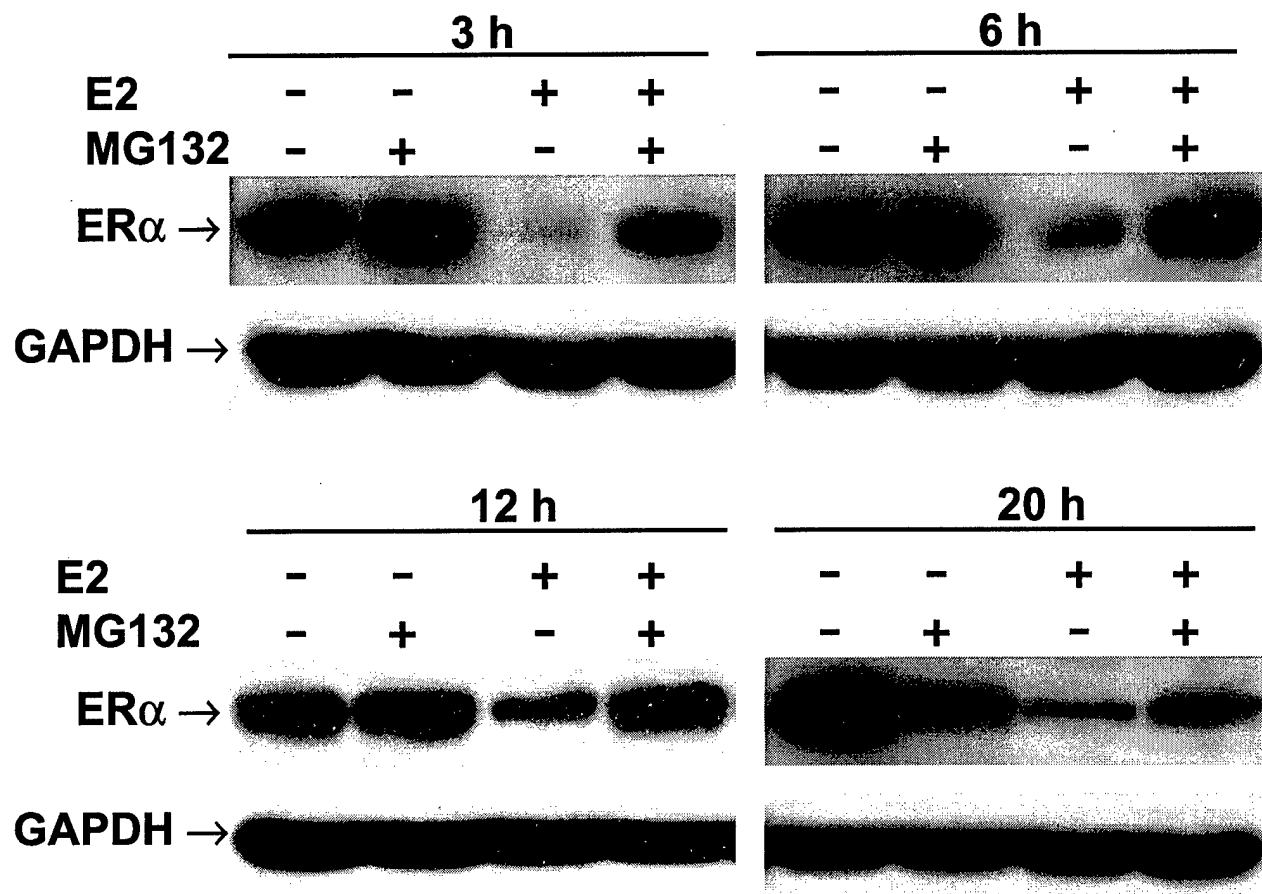


Fig 5E

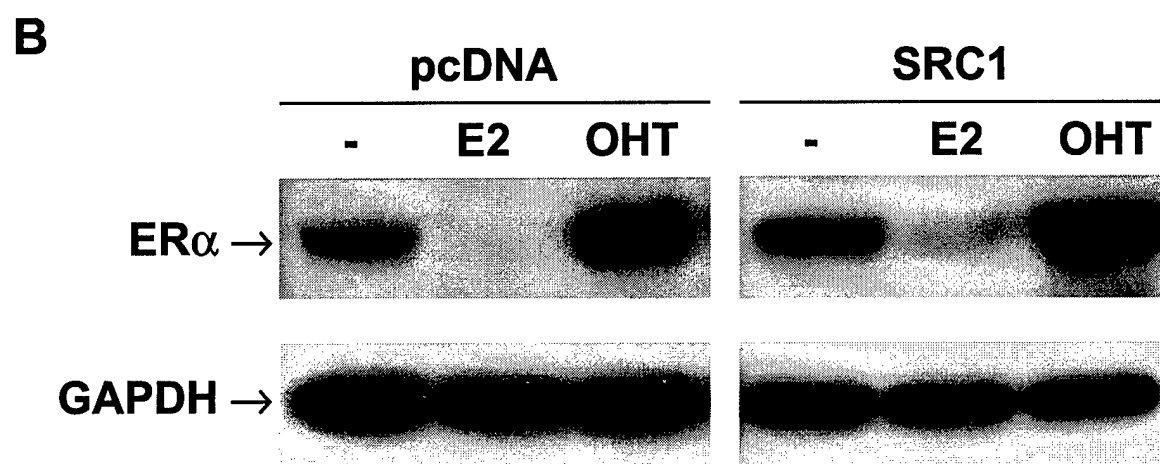
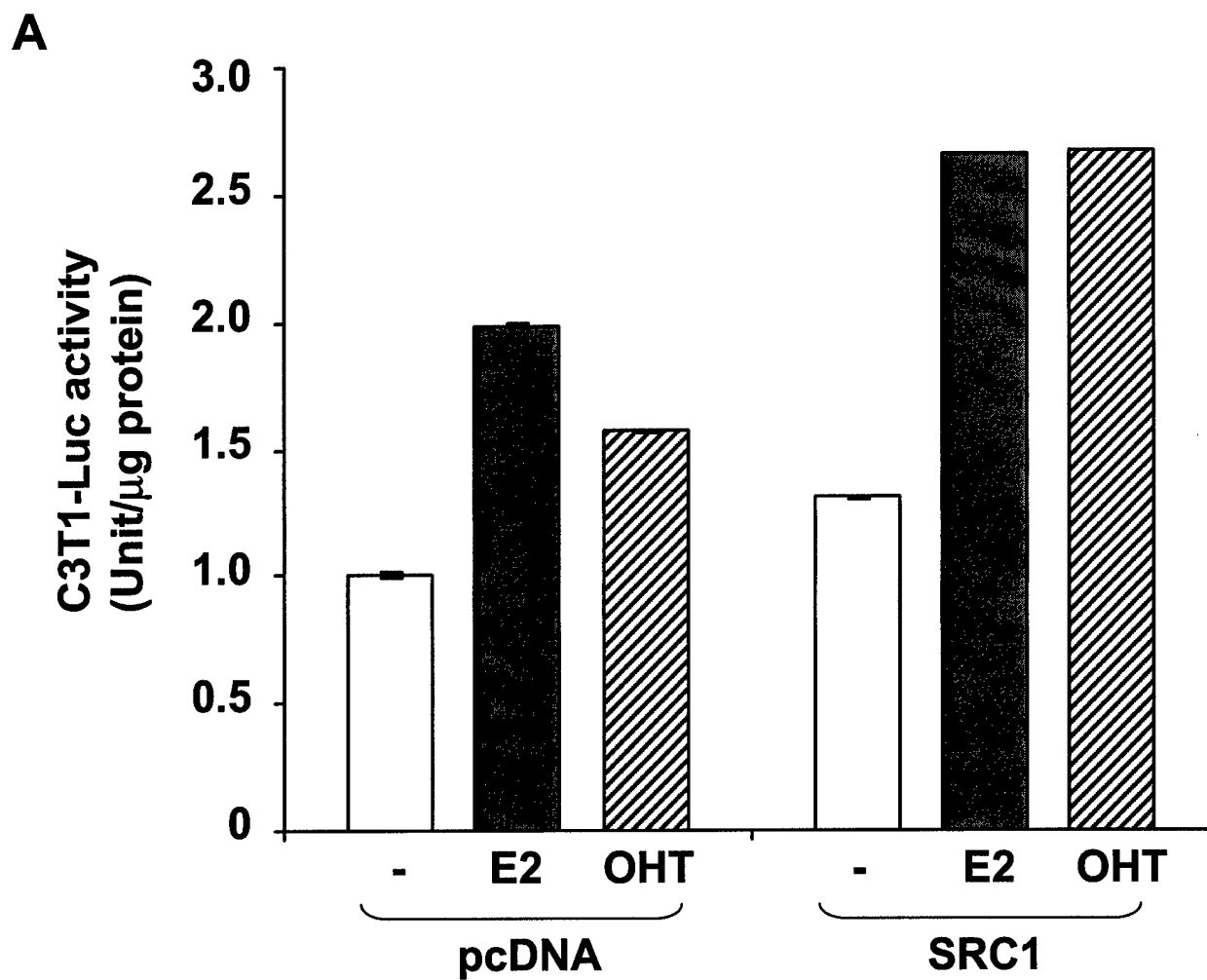


Fig 6

The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness.

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The hormone estrogen plays an important role in breast cancer development and progression. The actions of estrogen are mediated primarily by ER, a short-lived, ligand-activated transcription factor. Cellular turnover of ER is mediated primarily by the 26S proteasome, yet the functional consequence of receptor down-regulation and degradation on estrogen signaling is not clear. In the present study, the effect of inhibiting the 26S proteasome on ER-mediated gene transactivation was investigated. HeLa cells were transiently transfected with an ER-responsive chloramphenicol acetyltransferase reporter gene (ERE-vitellogenin-CAT) and various doses of ER expression vector (0.1 to 5 ng pSG5-ER/10⁵ cells). Twenty four hours later, cells were treated with proteasome inhibitor MG132 (1 μ M) for 1 h followed by treatment with 17 β -estradiol (E2, 10 nM) for 24 h. ER activity was determined by measuring CAT expression. A synergistic effect of MG132 on E2-induced CAT reporter expression was observed only in cells transfected with low levels of ER (0.1 - 1 ng pSG5-ER/10⁵ cells); furthermore, synergism between MG132 and E2 on ER transcriptional activity was inversely correlated with the level of ER expression. In the absence of ligand, MG132 increased ER-mediated transactivation by more than two fold. In the ER-positive human MCF7 breast cancer cell line transfected with ERE-vitellogenin-CAT, MG132 treatment increased both ligand-independent and -dependent ER transcriptional activity; however, enhancement of ER transactivation function by MG132 was less compared to HeLa cells expressing a low level of ER. Treatment of MCF7 cells with Geldanamycin (GA), an HSP90 inhibitor, caused rapid degradation of ER and decreased CAT reporter gene expression, but pretreatment with MG132 blocked GA-mediated ER down-regulation and restored ER transactivation activity. Collectively, these results suggest that by regulating ER protein levels, the 26S proteasome pathway restricts ER activity and thus cellular responsiveness to estrogen. Furthermore, in a stable MCF-7 cell line containing a disrupted NEDD8 pathway, higher steady-state levels of ER were observed and cell survival rate in the presence of the antiestrogen ICI 182,780 was greater compared to wild type MCF7 cells. Collectively, these results provide further evidence suggesting that the 26S proteasome pathway functions to restrict ER activity and consequently limit hormone responsiveness by regulating ER protein levels. Inhibition of breast cancer cell growth by ICI 182,780 is mediated in part by the ability of the drug to induce ER degradation, and our studies suggest that disruptions in the ER degradation pathway may confer cells growth advantage and provide a mechanism by which cancer cells acquire ICI 182,780 resistance.

The role of proteasome-mediated estrogen receptor- α (ER) degradation in estrogen responsiveness.

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Estrogen receptor-alpha (ER α) is a ligand-dependent transcription factor and mediator of physiological responses of tissues to 17 β -estradiol (E2). Binding of E2 to ER α rapidly downregulates receptor levels through targeted degradation by the proteasome. ER α turnover appears to be coupled to the transactivation ability of ER α , but proteasome inhibitors (e.g., MG132) interfere with the production of luciferase and β -galactosidase proteins, complicating the interpretation of studies using these reporter genes to show that inhibiting proteasome degradation inhibits ER α transcriptional activity. In the present study, the effect of inhibiting ER α degradation on receptor transcriptional activity was investigated using various ER α -responsive reporter constructs (ERE-vit-CAT, ERE-pS2-Luc) and ER α -negative HeLa cells transfected with varying amounts of ER α . Cells were treated with the proteasome inhibitor MG132, and the cellular responsiveness to E2 was examined. Proteasome inhibition enhanced E2-mediated transcriptional activity of an ER α -responsive CAT reporter. In cells transfected with low levels of ER α (0.1 - 1 ng pSG5-ER/10⁵ cells) and treated with MG132 and E2, a synergistic effect on ER α activity was observed, and a time course analysis showed that MG132 treatment prolonged ER α -mediated transcription. Consistent with this finding, ER α -mediated transactivation in transfected HeLa cells was prolonged by blocking receptor ubiquitination and degradation with Ubc12C111S, a dominant negative mutant of the ubiquitin-like NEDD8 conjugation enzyme. Treatment of MCF7 breast cancer cells, which endogenously express ER α , with MG132 increased E2-induced expression of both an ER α -responsive reporter gene and an endogenous ER α -target gene, pS2. Collectively, we demonstrate that the appropriate reporter gene is necessary to determine the relationship between proteasomal degradation and ER α transcriptional activity. Moreover, proteasomal degradation is not essential for ER α transactivation function, and ER α remains functional in the absence of an intact ubiquitin-proteasome system. Finally, our study shows that proteasomal degradation plays a key role in terminating ER α -mediated transcription.

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Code for the meeting: J8 (Nuclear receptor: steroid sisters)

Poster session

Uncoupling Estrogen Receptor- α Transcriptional Activity from Receptor Degradation

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The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; csFBS, dextran-coated charcoal-stripped fetal bovine serum; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; luciferase, firefly luciferase; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; SRC, steroid receptor coactivator; Ub, Ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin

Key Words: estrogen receptor, proteasome, transactivation, degradation

Abstract

Estrogen receptor-alpha (ER α) is a ligand-dependent transcription factor and mediator of physiological responses of tissues to 17 β -estradiol (E2). Binding of E2 to ER α rapidly down-regulates receptor levels through targeted degradation by the proteasome. ER α turnover appears to be coupled to the transactivation ability of ER α , but the functional impact of ligand-induced ER α degradation on cellular responses to E2 has not been fully established. In the present study, the effect of blocking the ubiquitin-proteasome pathway on ER α -mediated transcriptional response was investigated. In HeLa cells transfected with ER α , blocking both receptor ubiquitination and 26S proteasome-mediated turnover of ER α markedly increased E2-induced expression of an ER-responsive reporter gene. Time course studies further demonstrated that blocking ligand-induced degradation of ER α resulted in prolonged stimulation of E2-mediated gene transcription. In breast cancer MCF7 cells containing endogenous ER α , proteasome inhibition enhanced ER α -responsive reporter gene expression and expression of endogenous ER-target genes. In addition, in estrogen responsive endometrial cancer Ishikawa cells transfected with the SRC1 coactivator, 4-hydroxytamoxifen displayed full agonist activity and stimulated ER α -mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ER α transcriptional activity and functions to limit E2-induced transcriptional output.